Experiment diary

Week 1

14/09/2020 (Zichen and Bingyu)

- Grow E. coli (K12 MG1655) on agar plate, incubate at 37℃ overnight, gas burner is required

15/09/2020 (Zichen and Bingyu)

- Pick one colony from plate (gas burner required)
- Grow E. coli in LB culture at 37℃ overnight

16/09/2020 (Zichen and Bingyu)

- Measure the OD600 of each overnight culture, 1.723 and 1.715 (too low to start making cell lysate)
- Grow new batch of E. coli in LB at 37℃ overnight

17/09/2020 (Ziyue and Nan)

- Measure the OD600 of each overnight culture, 2.399 and 2.420
- Taken 1ml culture from the OD600 2.420 sample (gas burner required) and grow in 300ml M9 Minimal media at 37℃ overnight (160 rpm, at 37℃, incubate since 14:45)

18/09/2020 (Mengxi)

- Measure the OD600 of the overnight sample, 1.936 (17:00) [E. coli grow slowly in minimum culture, might require more initial bacteria added in the culture]
- Cell cultures were collected in six 50ml falcon tubes
- Cells were pelleted by spinning at 4500 RPM for 20 mins at 4℃
- Weight of each tube+ cell pellet: 64.175, 62.514, 63.005, 64.004, 62.968, 63.032 (g)
- Cell pellets were stored in -20℃

Week 2

21/09/2020 (Ziyue and Nan)

Cell lysis

- Place 6 tubes with pellet on ice
- Add 3ml non-ionic lysis buffer [0.1%v/v Triton X-100 (Sigma-Aldrich #T9284), 40 mM Tris base adjusted to pH 7.5 with HCl and 50 mg/mL lysozyme (Sigma-Aldrich #L6876)] to each pellet for cell resuspension
- Wash all tubes with 1ml non-ionic lysis
- Transfer all the resuspended cells into a new 50ml falcon tube (weight without cell: 12.757g, with cells: 32.693g)
- Add 0.5mg/ml lysozyme (stock conc: 50mg/ml) into cells (if don't need to be concentrated, use 1:1 as the amount of culture; if need to be concentrated, use 1:10 as the amount of culture)
- Keep the solution on ice (4℃) for 1h to allow the reaction fully act.
- Centrifuge at 4℃, 10000 x g for 20 mins. Collect supernatant in 20 1ml Eppendorf tubes, labelled from 1-20.
- Tube 1 and 2 were used for Bradford assay, tube 3 and 4 were used for runoff treatment

and then Bradford assay.

Runoff (nucleic acid degradation)

- Shake tube 3 and 4 at 160 rpm for 2h at 37℃
- Some white precipitations appeared at the bottom of the tube (? Might have some protein degradation)

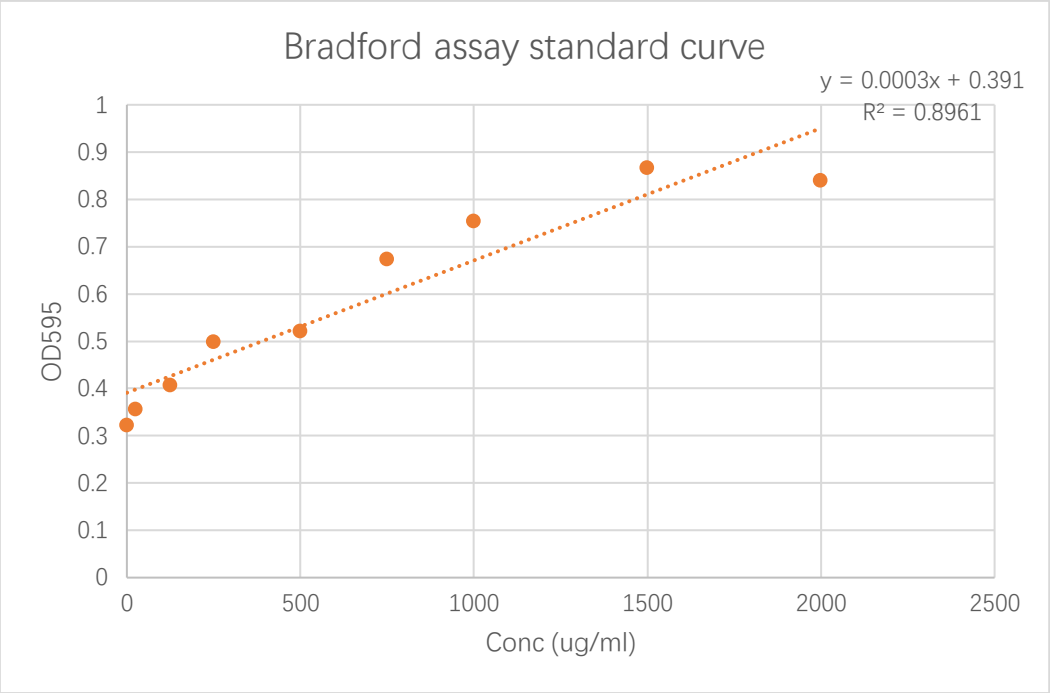
Braford assay of protein concentration in cell extract

- 5 ul of each standard or unknown sample into the appropriate microplate wells (e.g., Thermo Scientific™ Pierce™ 96-Well Plates, Product No. 15041).
- Add 250µL of the Coomassie Reagent to each well and mix with plate shaker for 30 seconds.
- Remove plate from shaker. For the most consistent results, incubate plate for 10 minutes at room temperature (RT).
- Measure the absorbance at or near 595nm with a plate reader.
- Subtract the average 595nm measurement for the Blank replicates from the 595nm measurements of all other individual standard and unknown sample replicates.
- Prepare a standard curve by plotting the average Blank-corrected 595nm measurement for each BSA standard vs. its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.

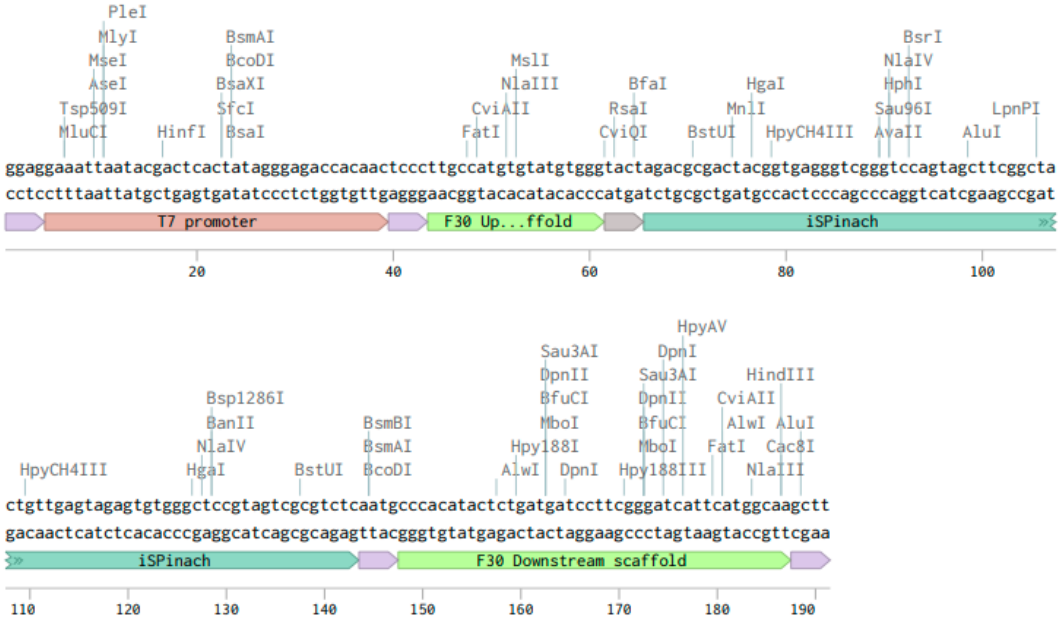
96 well plate layout:

Standard A triplicate	Standard I triplicate			Sample 1 triplicate					
Standard B triplicate				Sample 2 triplicate					
Standard C triplicate									
Standard D triplicate				Sample 3 triplicates					
Standard E triplicate				S4					
Standard F triplicate					Sample 4				
Standard G triplicate									
Standard H triplicate									

0.314	0.314	0.341	0.882	0.799	0.838	1.096	1.052	0.962			
0.346	0.344	0.379				1.085	1.09	1.405			
0.458	0.364	0.398									
0.506	0.53	0.462				1.157	1.226	0.875			
0.557	0.516	0.492				1.183					
0.705	0.64	0.675					1.144	1.211			
0.791	0.744	0.725									
0.886	0.88	0.833									



Sample	Ave reading	Concentration (ug/ml)	
Standard A	0.839666667	2000	
Standard B	0.8663333	1500	
Standard C	0.7533333	1000	
Standard D	0.6733333	750	
Standard E	0.5216667	500	
Standard F	0.4993333	250	
Standard G	0.4066667	125	
Standard H	0.3563333	25	
Standard I	0.323	0	
Sample 1	1.036666667	2153	2413
Sample 2	1.193333333	2673.333	
Sample 3	1.086-0.323=0.763	1240	1395
Sample 4	1.179333333-0.323=0.856	1550	



Oligos phosphorylation

Amount of each parts

	Amount (nmol/mg)	Tm (℃)	Mw (mg/umol)
T7 Promoter F	20.4/ 0.25	62.2	12.3
T7 Promoter R	29.1/ 0.35	62.2	12.3
F30 up F	17.4/ 0.12	59.5	6.9
F30 up R	28/ 0.19	56.4	6.8
Aptamer F	5/ 0.13	73.1	26
Aptamer R	1.6/ 0.04	73.4	25
F30 down F	22.9/ 0.31	67.3	13.5
F30 down R	16.5/ 0.22	67.3	13.3

1. Spin the tubes containing the lyophilized oligonucleotides briefly (30 s, 2000 g) to ensure that the pellet is at the bottom of the tube.
2. Add nuclease free water to obtain a final concentration of 100 µM. Mix by flicking the tube, and spin briefly (30 s, 2000 g) to ensure that all liquid is at the bottom of the tube. Place the tubes on ice.
3. To phosphorylate oligonucleotides, combine the following in a 0.2 ml clear PCR tube:

Components	Stock conc	Volume (ul)
Forward part	100 μM	10
Reverse part	100 μM	10
T4 PNK buffer (NEB)	10x	2.5
T4 Polynucleotide Kinase	10000 U/ml	0.25
ATP	10 mM	2.5

4. Mix by flicking the tubes, and spin briefly (30 s, 2000 g) to return all liquid to the bottom of the tube. Place the tubes in a heat-block and incubate for 30 min at 37°C.
5. To anneal, place the tubes with the phosphorylated oligonucleotides in a heat-block at 95°C. Slowly cool down the block from 95°C by turning off the heat-block, and let it cool down to room temperature.
6. Tubes were stored in -20℃

[4 tubes in the end contain phosphorylated and annealed double stranded each part]

22/09/2020 (Nan and Ziyue)

1. Each part was added to a 1.5 ml microcentrifuge tube on ice as following for ligation:

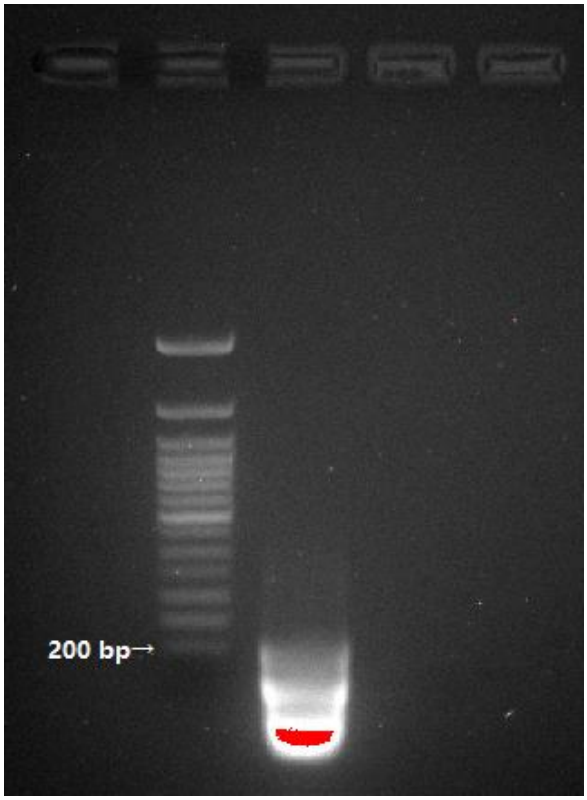
	Concentration	Volume
Promoter	40μM	4μl
F30 up	40μM	4μl
Aptamer	40μM	4ul
F30 down	40μM	4μl

T4 ligase buffer (+ATP)	10x (10mM)	3µl
T4 ligase	400000 U/ml	1.5µl
ATP	10mM	1.5µl

2. Mix by flicking the tubes, and spin briefly (30 s, 2000 g) to return all liquid to the bottom of the tube. Incubate at 16°C for 1:10 h.
3. Place the tubes in a pre-warmed heat-block (or water bath) and incubate at 65°C for 10 min to inactivate T4 DNA ligase. Spin the tubes briefly (30 s, 2000 g), and place on ice. Store at -20°C. Before use, defrost thoroughly on ice and spin the tubes briefly (30 s, 2000 g). Keep the tubes on ice when in use.
4. To check the quality of the Clips, analysis by 2% **agarose gel.**

	Sample	Dye buffer (6x)
50bp DNA ladder	5ul	
Sample	5ul	1ul

5. Run at **90v** for **40mins.**



Observe: no obvious band at ~200 bp, thus the ligation did not work. Might because the ligation time is not enough

Solution: increase the ligation at 16°C to 13h (overnight)

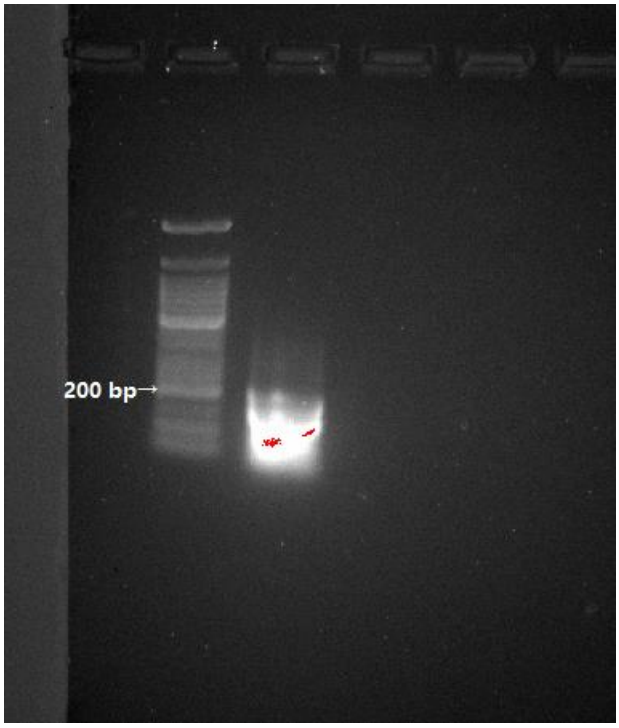
1. Within the same tube, add extra

Components	Volume
T4 buffer	3ul
T4 ligase	1.5ul
ATP	1.5ul

2. In PCR machine, incubate at 16°C for 13 h, 65°C for 10 min and store at 4°C.

23/09/2020 (Nan and Ziyue)

2% agarose gel was used to check the product from longer ligation (13h)



We found the aptamer sequence on the bottle is not complete, thus, we decided to perform a PCR for aptamer to obtain the whole length product.

PCR reaction system:

	Concentration	Volume/μl
Phosphorylated and annealed primers	40μM	0.5
Phusion High Fidelity Master Mix buffer	2x	12.5
Nuclease-free buffer		12
Total volume		25

PCR protocol:

	T/°C	t
	95	5 min
7 cycles	95	20 s
	54	10 s
	72	10 s
35 cycles	95	20 s
	72	30 s

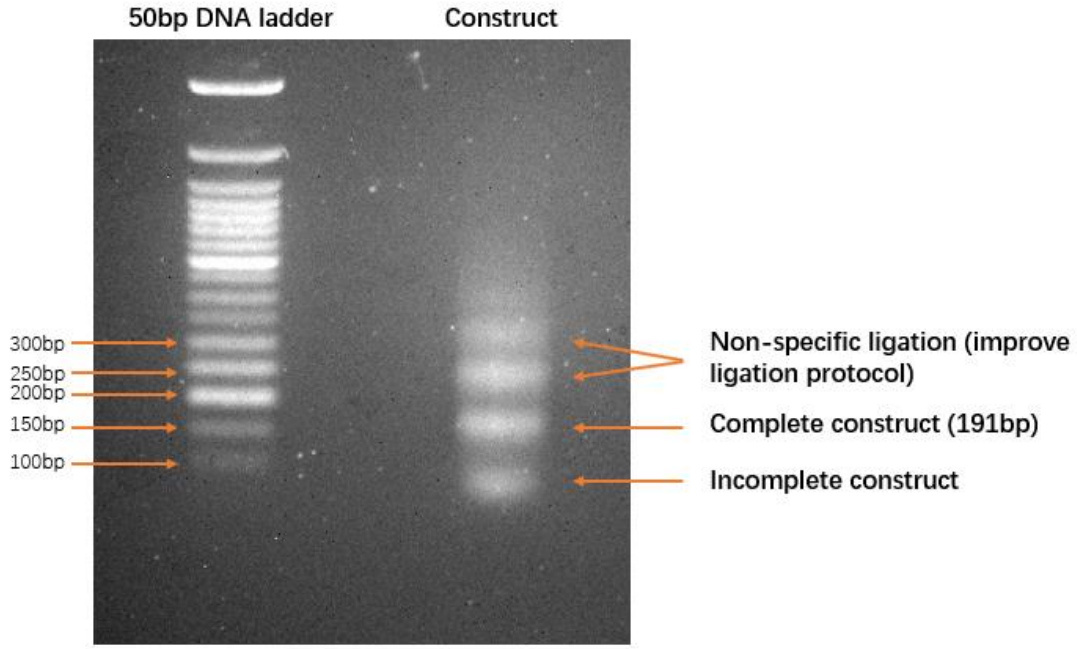
	72	5 min
	4	Infinity

After the PCR, the ligation reaction was performed as follow:

	Concentration	Volume
Promoter	40μM	0.5μl
F30 up	40μM	0.5μl
Aptamer	40μM	Whole PCR reaction system
F30 down	40μM	0.5μl
T4 ligase buffer (+ATP)	10x (10mM)	1μl
T4 ligase	400000 U/ml	0.5μl

1. Mix by flicking the tubes, and spin briefly (30 s, 2000 g) to return all liquid to the bottom of the tube.
2. PCR machine was set to incubate at 16°C for 13 hours, followed by 65°C for 10 min to inactivate T4 DNA ligase and finally stored in 4°C.
3. Analyze the construct by 2% agarose gel, 90V, 40mins

	Sample	Dye buffer (6x)
50bp DNA ladder	2.5ul	
Sample	2ul	0.4ul



Result: there are 4 bands shown on the gel as labeled. There might have some non-specific ligation due to long incubation time?

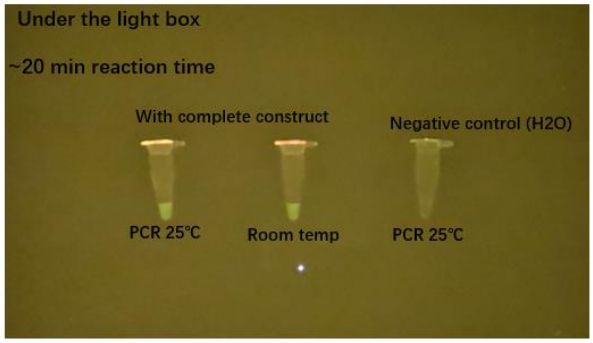
24/09/2020 (Nan and Ziyue)

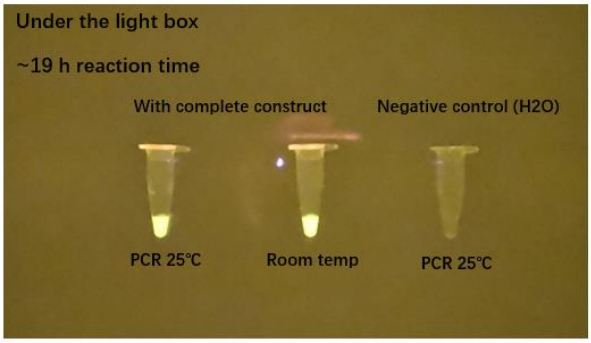
In vitro transcription

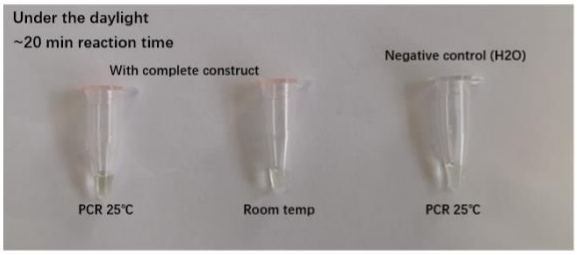
Reaction system:

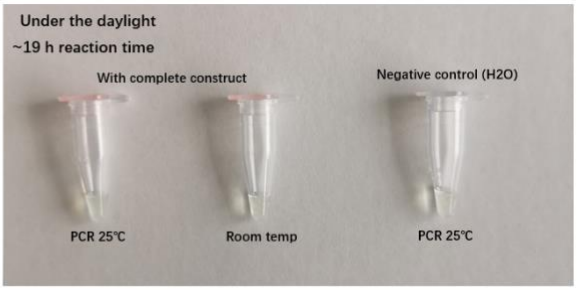
		Experiment group	Positive control	Negative control
	Stock concentration	V	V	V
Sample		1µl(<5ng/µl)	1µl (T7 construct form Mengxi)	0
Nuclease-free water		13.25µl	13.25µl	14.25µl
OTRDB	10x	2µl	2µl	2µl
DFHBI Fluorophore	200µM	1µl	1µl	1µl
NTPs	20mM	2µl	2µl	2µl
T7 RNAP	50000 U/ml	0.75µl	0.75µl	0.75µl

The above system has been divided into two groups, one is incubated at room temperature and the other is incubated at 25℃ for 20min.
Fluorescence were imaged under a Safe Imager™ Blue-Light Transilluminator (Invitrogen) with an amber filter unit and a phone camera









Time difference for the observable fluorescence for the room temperature and 25℃ incubated sample are not that different, which are all around 20 mins.

Reflect of previous experiments

- The aptamer sequence is actually complete, thus the PCR reaction only filled up the 4 bases fusion site. Not sure why the in vitro transcription worked, might be due to the incomplete PCR…..
- Shorten the overall ligation time to eliminate the unspecific ligation

Week 3

28/09/2020 (Nan and Ziyue)

The ligation protocol was improved on this day.

Ligation

Reaction system:
The ligation reaction this time was done in stepwise fashion, phosphorylated and annealed Promoter + F30 up scaffold and Aptamer + F30 down scaffold were ligated parallel in separate tubes. Then 5ul of each system were mixed and incubated at 16℃ for 1h.

Separately: (promoter+F30 up; aptamer+F30 down)

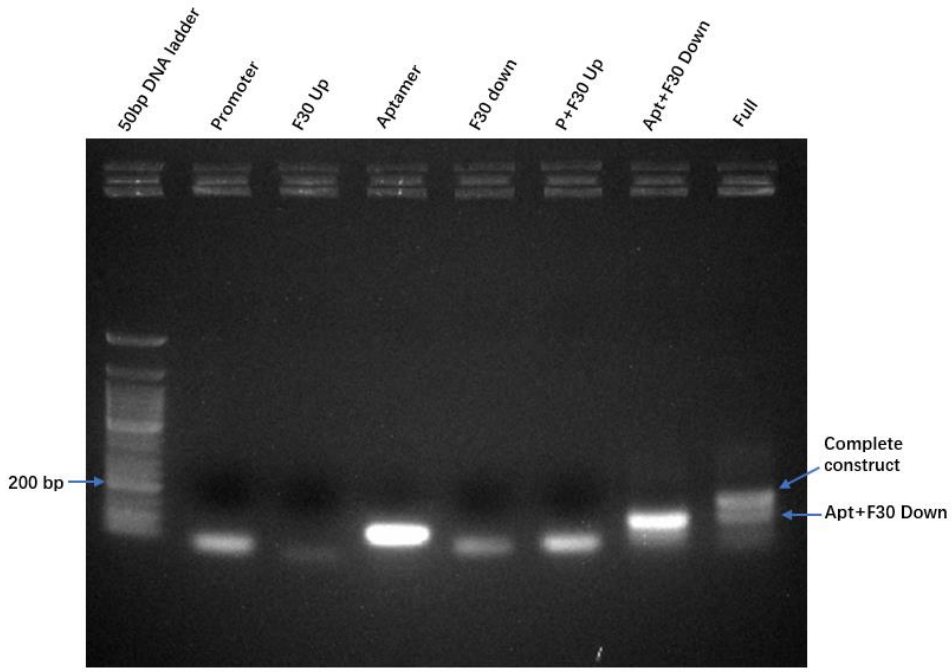
	Concentration	Volume
Upstream clip	40μM	2μl
Downstream clip	40μM	2μl
T4 ligase buffer (+ATP)	10x (10mM)	1μl
T4 ligase	400000 U/ml	0.5μl
Nuclease free water		4.5μl

Final concentration of whole-length construct: 8μM. Total volume: 10μl
Flick tube and spin for 30s.
Incubate at 16℃ for 1h

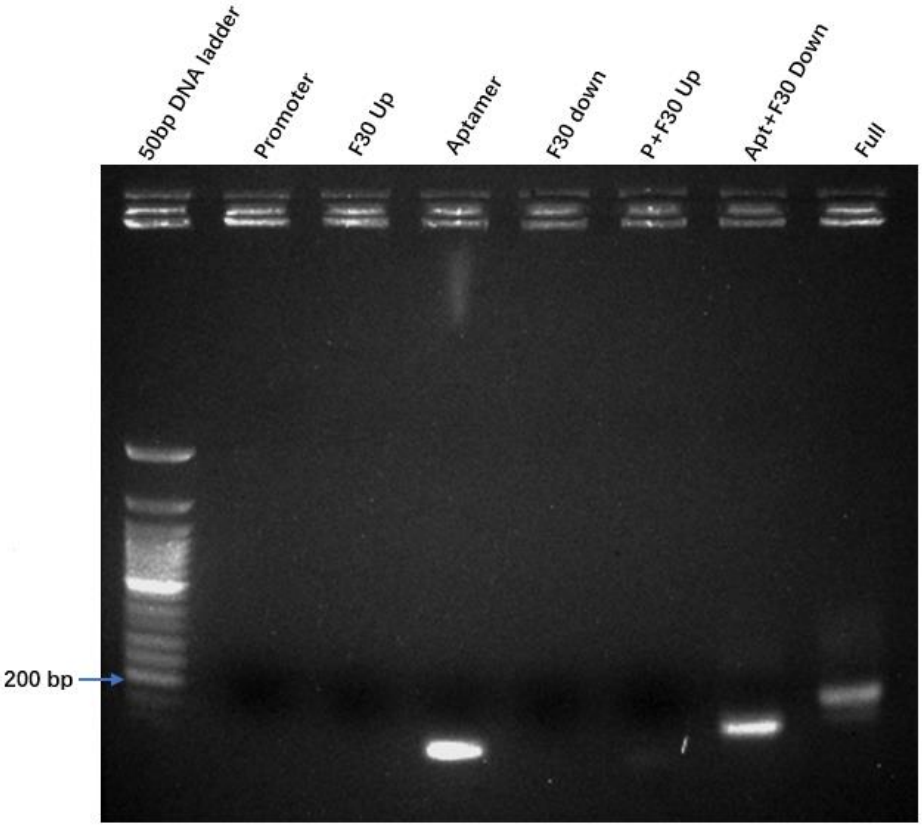
Mix two reaction systems together (each 5μl). Incubate at 16℃ for 1h
Then, incubate at 65℃ for 10min for ligase inactivation.

Agarose gel electrophoresis

2% gel: 50ml TAE+1g agarose+0.5μl SYBR safe (10000x)
50bp ladder: 5 μl;
Sample:
Promoter: 82 ng/lane
F30 up: 46 ng/lane
Aptamer: 174 ng/lane
F30 down: 90 ng/lane
Promoter+F30 up: 154 ng/lane
Aptamer+F30 down: 316 ng/lane
Full length: <235 ng/lane
90V, 30min or 40min



90V 30 mins



90V 40 mins

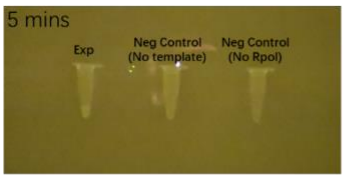
40 mins is too long, samples ran off the gel

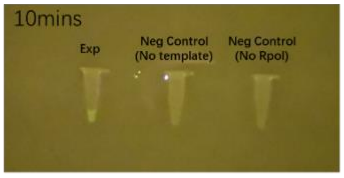
IVT

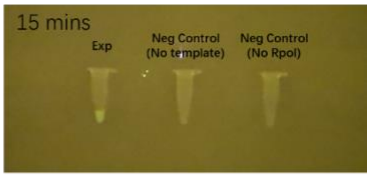
Reaction system:

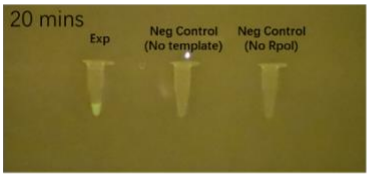
	Experimental group		Positive control	Negative control	
			with determined template	without template	without RNAP
	C	V	V	V	V
Sample	Total amount in the system should <100ng	1μl(~11.7ng)	1μl	0	1μl
Nuclease-free water		13.25μl	13.25μl	13.25μl	14μl
OTRDB	10x	2μl	2μl	2μl	2μl
DFHBI Fluorophore	200μM	1μl	1μl	1μl	1μl
NTPs	20mM	2μl	2μl	2μl	2μl
T7 RNAP		0.75μl	0.75μl	0.75μl	0

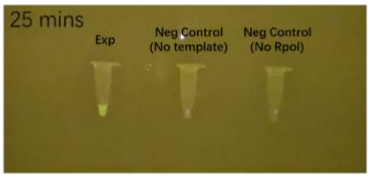
Incubate in RT

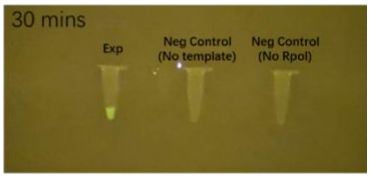


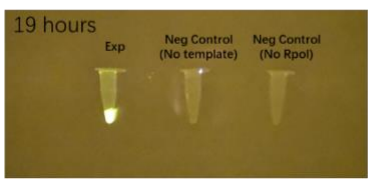


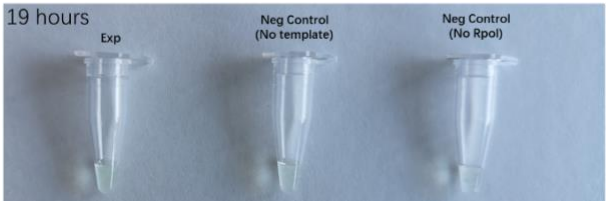












There is a problem with the positive control construct, so no fluorescence for the positive control.

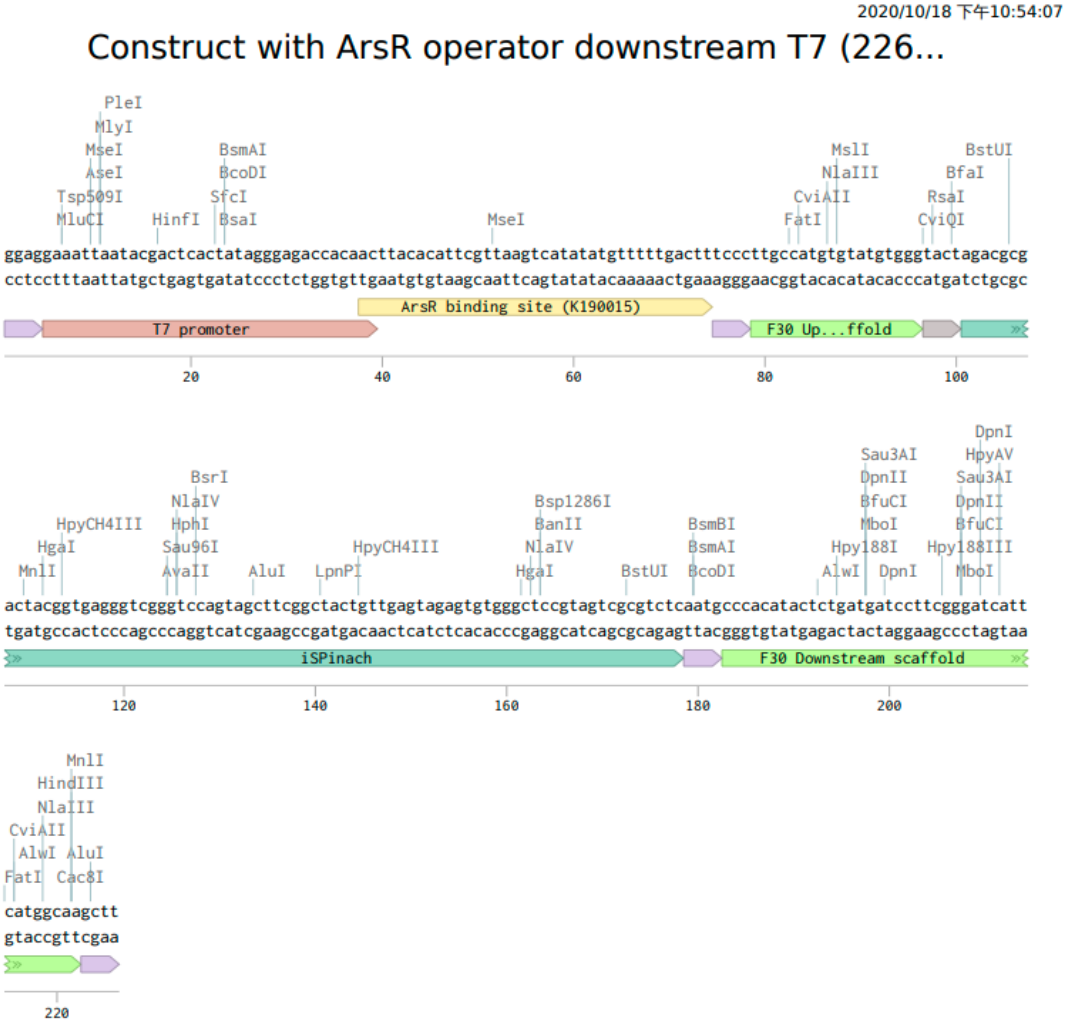
29/09 to 02/10

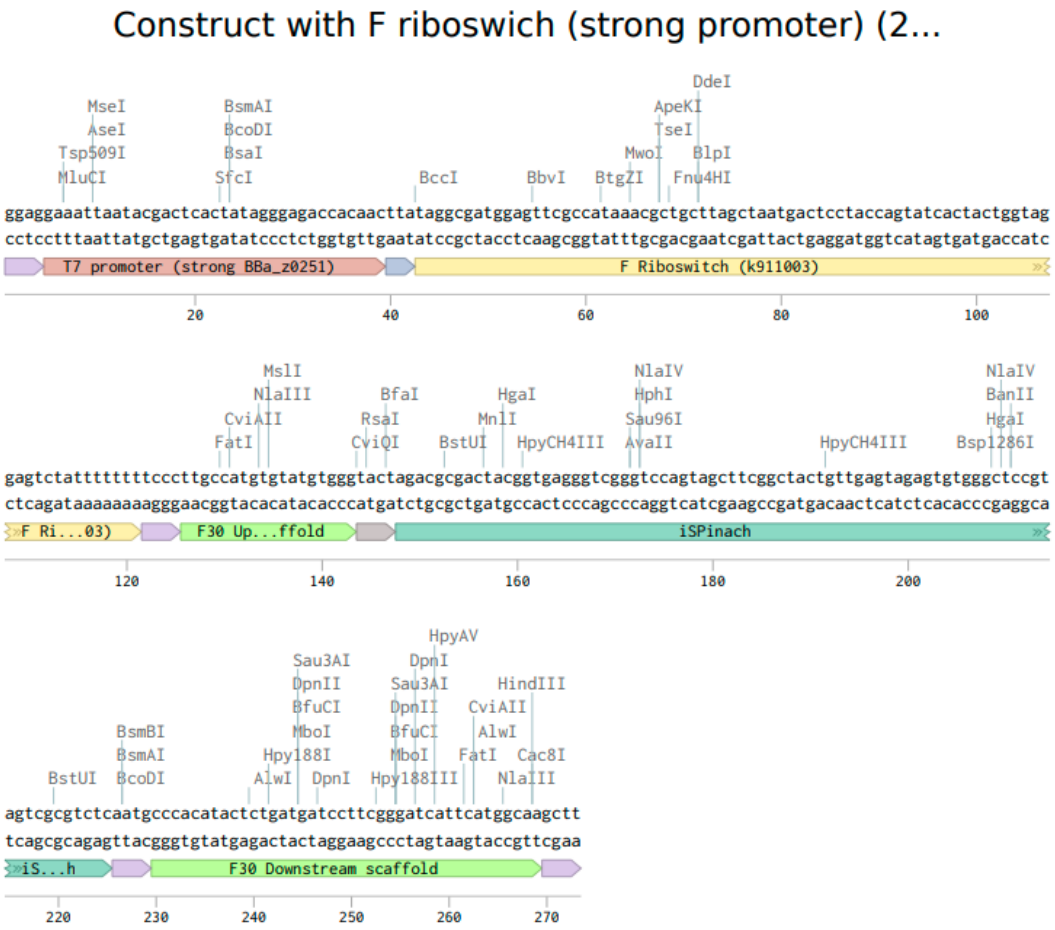
Designing and order new DNA templates

Week 4

05/10/2020 (Nan and Ziyue)

A new batch of construct has arrived. Which are promoters consisting of ArsR operator downstream of the T7 promoter and fluoride riboswitch downstream of the T7 promoter.





Parts

T7_ArsR Forward	100 nmol = 2.28 mg Tm = 66.6℃
T7_ArsR Reverse	80.9 nmol = 1.85 mg Tm = 66.2℃
Fluoride riboswitch upstream Forward	25.7 nmol = 0.46 mg Tm = 67.3℃
Fluoride riboswitch upstream Reverse	89.6 nmol = 1.78 mg Tm = 67.1℃
Fluoride riboswitch downstream Forward	112.4 nmol = 2.2 mg

	Tm = 67.2℃
Fluoride riboswitch downstream Reverse	27.4 nmol = 0.48 mg Tm = 67.3℃

*Because of the limitation in DNA ordering size, the T7_F riboswitch has been divided in to upstream and downstream part.

Phosphorylation and annealing

1. Spin the tubes containing the lyophilized oligonucleotides briefly (30 s, 2000 g) to ensure that the pellet is at the bottom of the tube.
2. Add nuclease free water to obtain a final concentration of 100 µM. Mix by flicking the tube, and spin briefly (30 s, 2000 g) to ensure that all liquid is at the bottom of the tube. Place the tubes on ice.
3. To phosphorylate oligonucleotides, combine the following in a 0.2 ml clear PCR tube:

Components	Stock conc	Volume (ul)
Forward part	100 µM	10
Reverse part	100 µM	10
T4 PNK buffer (NEB)	10x	2.5
T4 Polynucleotide Kinase	10000 U/ml	0.25
ATP	10 mM	2.5

Volume of the system is 25.25 ul. Concentration of the construct is 40 µM.

4. Mix by flicking the tubes, and spin briefly (30 s, 2000 g) to return all liquid to the bottom of the tube. Place the tubes in a heat-block and incubate for 30 min at 37°C.
5. To anneal, place the tubes with the phosphorylated oligonucleotides in a heat-block at 95°C. Slowly cool down the block from 95°C by turning off the heat-block, and let it cool down to room temperature.

[**3 tubes in the end consisting of double stranded T7_ArsR, F riboswitch upstream and F riboswitch downstream**]

Ligation

For the **first step two-part ligation**, four reactions were established

1) **T7_ArsR + F30 upstream**
2) **iSpinach + F30 downstream**
3) **riboswitch upstream + riboswitch downstream**
4) **F30 upstream + iSpinach-F30 downstream**

For the first 3 reactions, the following are added:

	Concentration	Volume
Upstream clip	40 µM	4 µl
Downstream clip	40 µM	4 µl
T4 ligase buffer (+ATP)	10x (10 mM)	2 µl
T4 ligase	400000 U/ml	1 µl
Nuclease free water		9 µl

Final concentration of whole-length construct: 8 µM. Total volume: 20 µl

For the reaction 4, the iSpinach-F30 downstream construct was build last time, with the concentration of 8 uM. Thus, the following were added as follow:

	Concentration	Volume
Upstream clip	40 µM	1 µl
Downstream clip	8 µM	5 µl
T4 ligase buffer (+ATP)	10x (10 mM)	1 µl

T4 ligase	400000 U/ml	0.5 µl
Nuclease free water		2.5 µl

Final concentration of whole-length construct: 4 µM. Total volume: 10 µl

Flick tube and spin 30s.
Incubate at 16℃ for 1h

For the **last step full length construct ligation**:
10 ul were taken from reaction 1) and 2) respectively and mixed into a new tube
5 ul reaction 3) and 10ul of reaction 4) were mixed into a new tube

Incubate at 16℃ for 2h. Then, incubate at 65℃ 10min for ligase inactivation. 4℃ overnight.

Product:

T7_ArsR full length construct with concentration 4 uM.

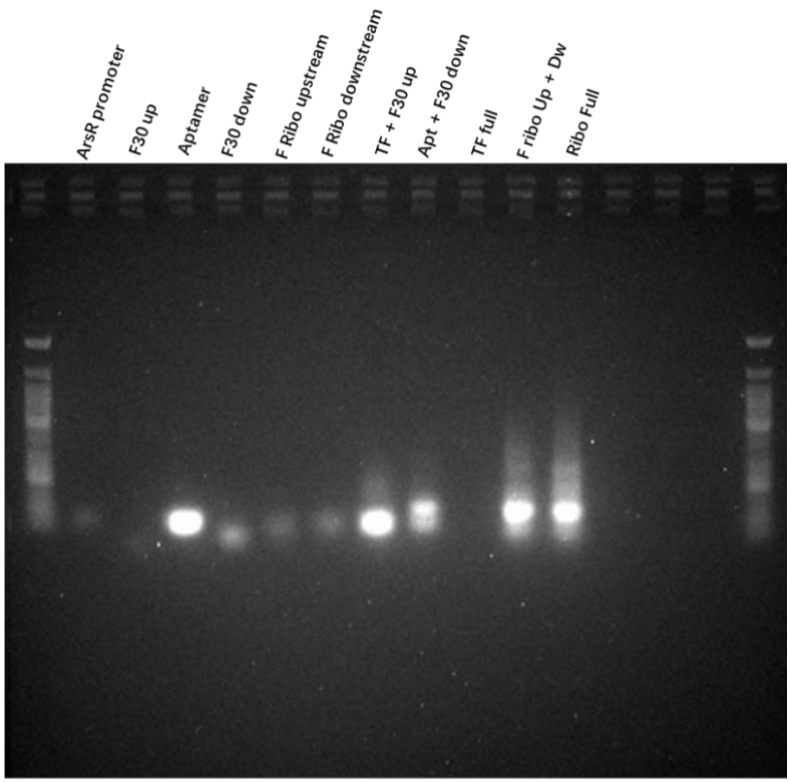
T7_F riboswitch full length construct with concentration 2.7 uM

06/10/2020 (Nan and Ziyue)

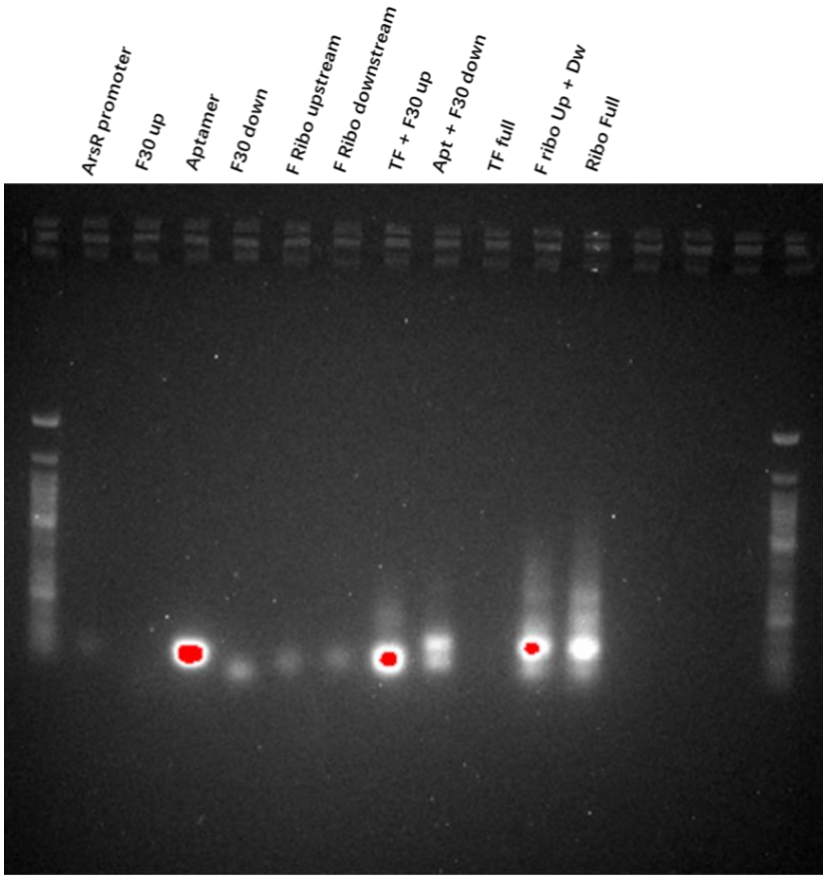
2% agarose gel

2% gel: 50ml TAE+1g agarose+0.5µl SYBR safe (10000x)
50bp ladder 1.2 ul;
Sample:
T7_ArsR: 50 ng/lane
F30 up: 50 ng/lane
Aptamer: 50 ng/lane
F30 down: 50 ng/lane
Riboswitch up: 50 ng/lane
Riboswitch down: 50 ng/lane
T7_ArsR + F30 up: 50 ng/lane
Aptamer + F30 down: 50 ng/lane
Riboswitch up + down: 50 ng/lane
F30 up + aptamer + F30 down: 50 ng/lane
T7_ArsR full length: 50 ng/lane
Riboswitch full length: 50 ng/lane

90V, 30 mins and 35 mins



30 mins



35 mins

The resolution for the gel is quite low, there seems to have a band ~200bp that represents the F riboswitch full construct. However, the ArsR ligated construct is missing… We proceeded with IVT anyways, to see whether there are any successfully ligated constructs.

NaF solution dilution

Mw = 41.99 g/mol

0.4225 gram of solid was added into 50 ml nuclease-free water to make 0.2 mol/L stock.

0.5 ul was taken and dilute into 10 ml to make 0.01 mol/L stock.

0.1 ul was taken and dilute into 10 ml to make 100 umol/L stock.

IVT

Four in vitro transcription were performed

1) ArsR construct without metal (negative control)
2) ArsR construct with 7 µM As (III)
3) Riboswitch construct without metal (negative control)
4) Riboswitch construct with 7 µM NaF

The following were added, the ones without metals, their volume were filled up with water

Sample	Stock conc	Final conc	Volume (µl)
OTRDB buffer	10X	1X	1.5
DFHBI fluorophore	200 µM	10 µM	0.75
NTP	20 mM	2 mM	1.5

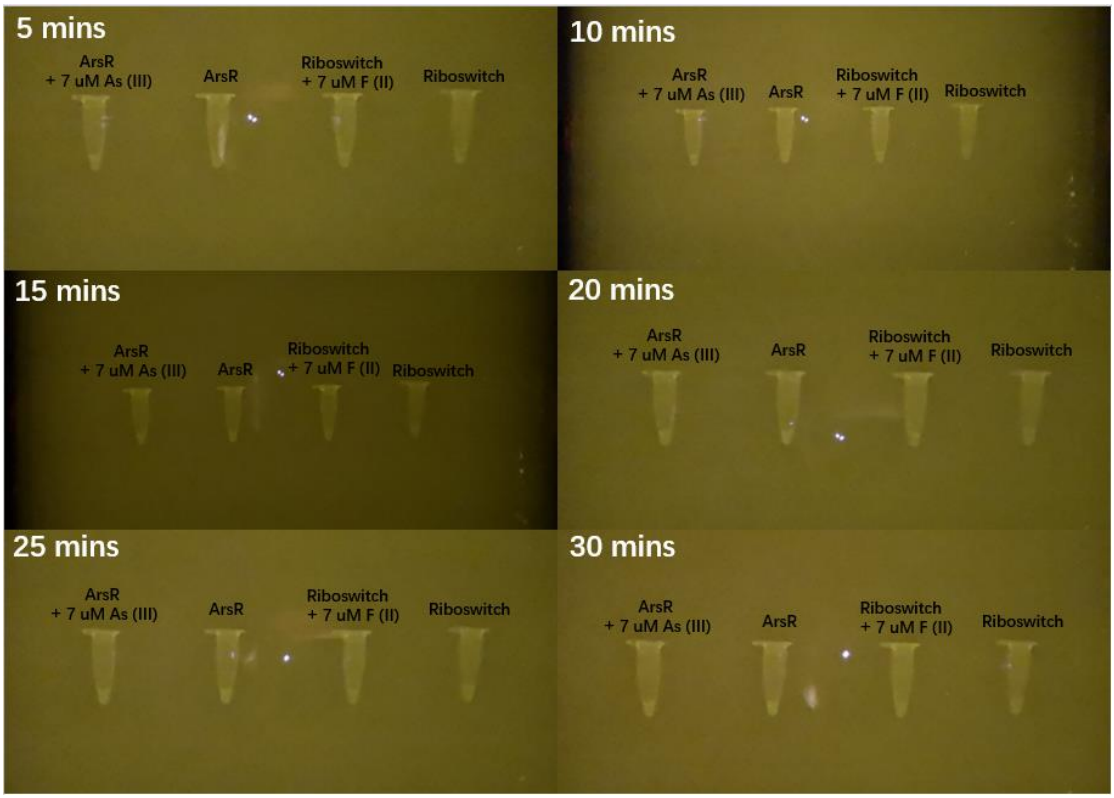
T7 Rpol	50000 U/ml	1.5 U/ul	0.45
Cell extract		30% v/v	4.5
Template	ArsR 4 µM	112 ng	0.2·
	F ribo 2.7 µM	137 ng	0.3
Metal	2 µM	0.3 µM	2.25
	7 µM	2 µM	4.3
	100 µM	7 µM	1.05
Nuclease-free water			Fill up to **15** ul

The definition of U here indicates the unit, 1nmol/50ul*60mins, which be simplified as 3.3 *10^-7 M/min. In our system, the conc of TF and riboswitch DNA template added is 5.3 * 10^-8, which is 10 times less than the T7 Rpol activity.

For the ArsR transcription factor construct, cell extract was added first, then DNA template, then T7 Rpol, metal was added the last. This ideally give time for TF binding to its operator site on the template.

For the fluoride riboswitch, cell extract and DNA construct was added the first, then metal ions, T7 was added the last. Because the riboswitch recognition for the metal ions are co-transcriptional. Therefore, metal ions need to be present while transcribing.

Results:



Reflect:

Not much difference can be observed between the experiment and negative control groups. Might be due to several reasons.

1) For the TF construct, the cell extract and DNA template need to be incubated for longer time to allow as much binding as possible before adding T7 RNA polymerase, to reduce the leaky transcription.
2) For the TF construct, the DNA template might be too much, reduce the amount next time.
3) For riboswitch construct, the cell extract was added by accident this time, which might interfere somehow with the performance of the riboswitch. No cell extracts next time.
4) Remember positive control (T7 only construct) next time!!!
5) Calculate the exact No of DNA template.

6) Using a plate reader for precise fluorescence data

07/10/2020 (Nan)

IVT for ArsR and F ribo construct, adding 50ng DNA template and 7 uM metal.

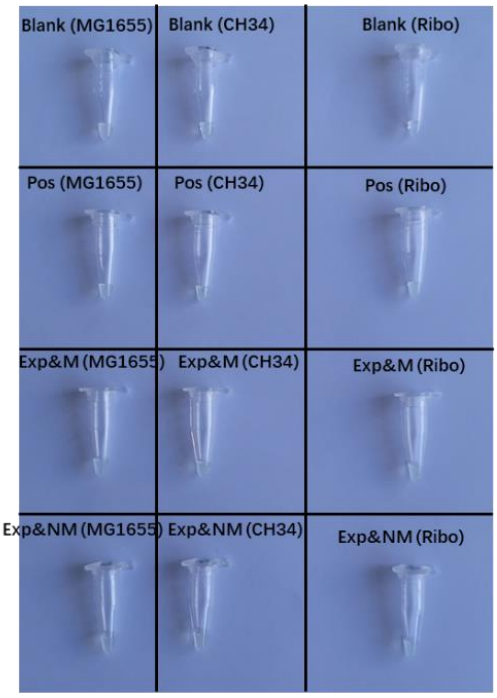
Sample	Stock conc	Final conc	Blank (µl)	Pos ctl (T7 construct)	TF + 7 uM As	TF only
OTRDB buffer	10X	1X	1.5	1.5	1.5	1.5
DFHBI fluorophore	200 µM	10 µM	0.75	0.75	0.75	0.75
NTP	20 mM	2 mM	1.5	1.5	1.5	1.5
T7 Rpol	50000 U/ml	1.5 U/ul	0.45	0.45	0.45	0.45
Cell extract		30% v/v	4.5	4.5	4.5	4.5
Template	ArsR 1 µM	50 ng		T7: 0.424	0.358	0.358
	100 µM	7 µM	1.05	1.05	1.05	
Nuclease-free water		Fill up to **15** µl	5.25	4.826	4.892	5.942

Sample	Stock conc	Final conc	Blank (µl)	Pos ctl (T7 construct)	Ribo + 7µM As	Ribo only
OTRDB buffer	10X	1X	1.5	1.5	1.5	1.5

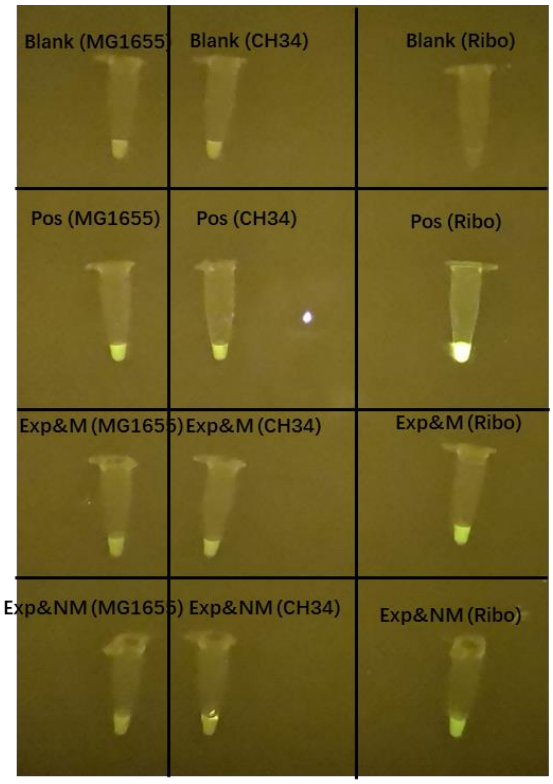
DFHBI fluorophore	200 µM	10 µM	0.75	0.75	0.75	0.75
NTP	20 mM	2 mM	1.5	1.5	1.5	1.5
T7 Rpol	50000 U/ml	1.5 U/ul	0.45	0.45	0.45	0.45
Template	F Ribo 1 µM	50 ng		T7: 0.424	0.296	0.296
	100 µM	7 µM	1.05	1.05	1.05	
Nuclease-free water		Fill up to **15** µl	9.75	9.326	9.454	10.504

Results:

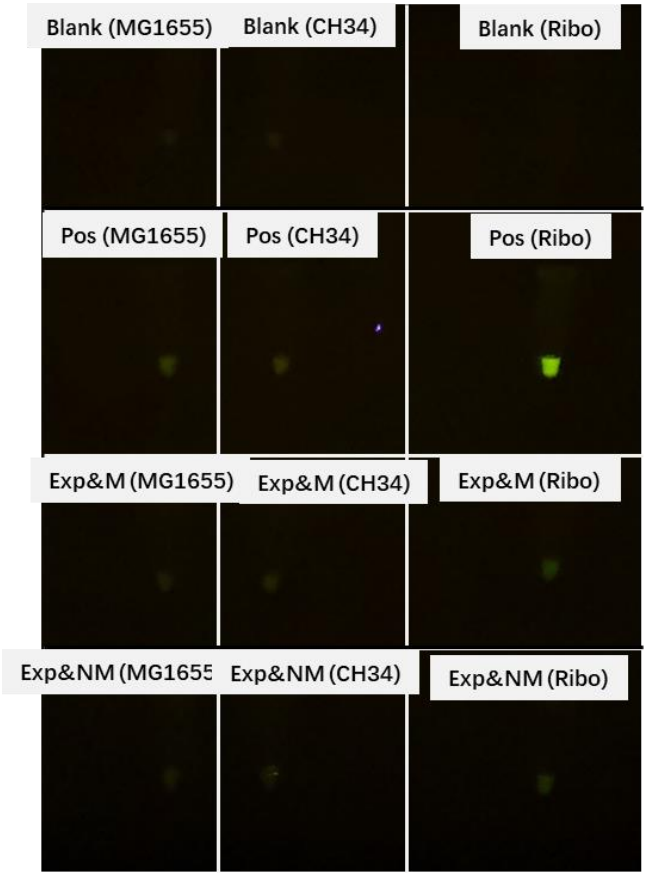
Fluorescence after 19 h reaction



Under the natural light

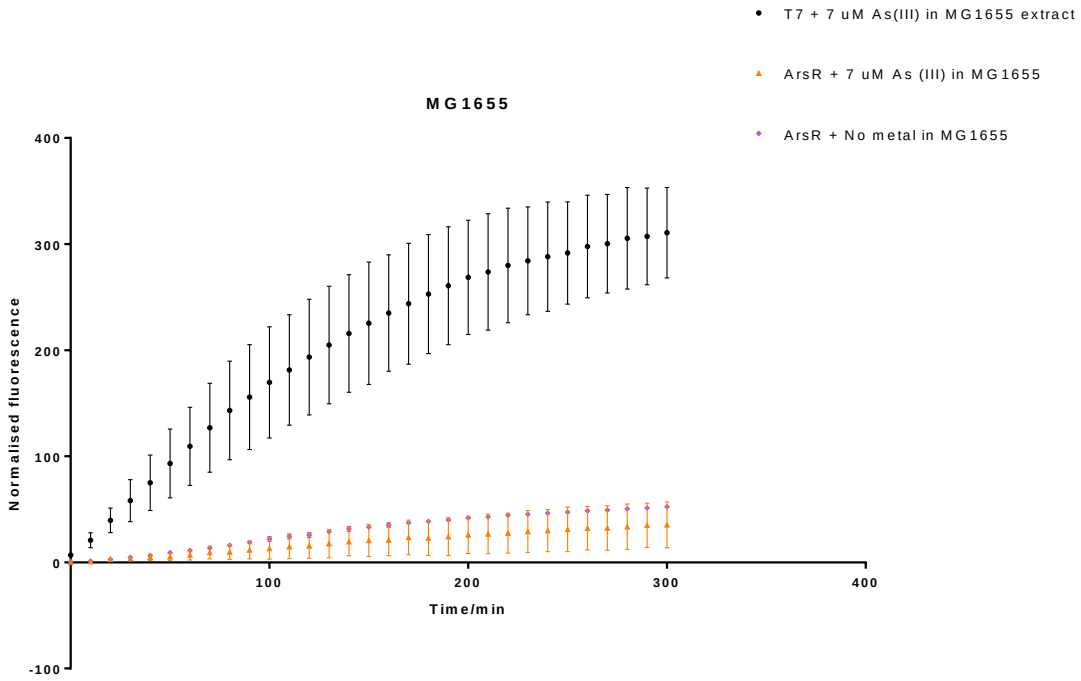


Under the blue light



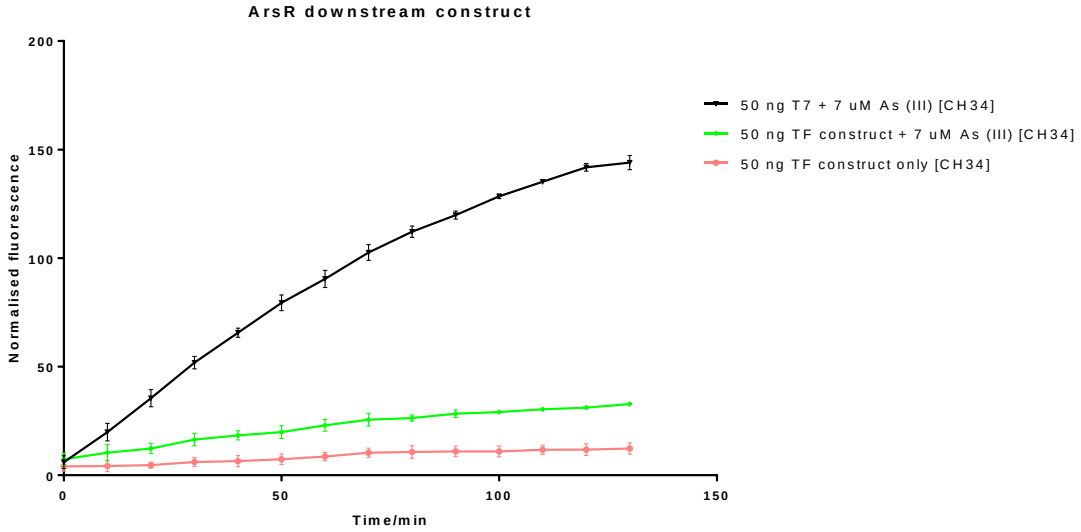
In the dark

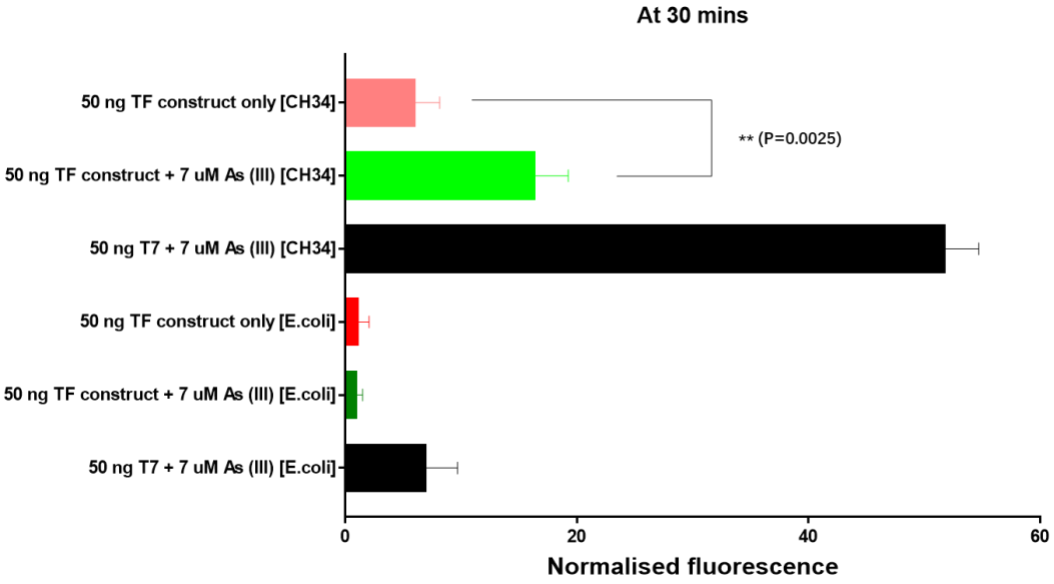
Graph draw below is based on two repeats only, since one repeat in Positive control group has large variation, thus only two repeats were taken from all experimental groups.

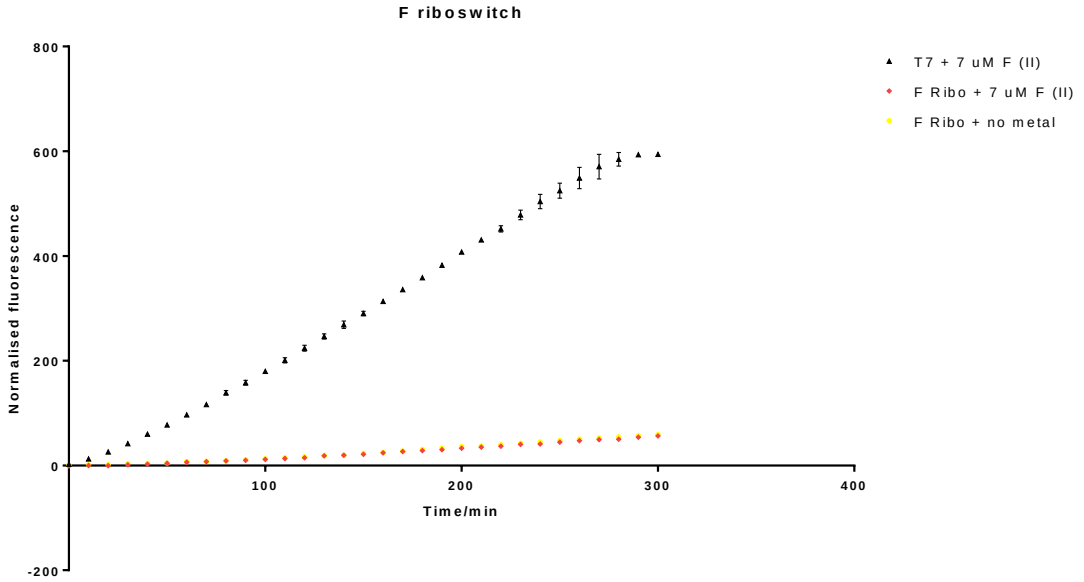


Difference in fluorescence can be observed in systems with and without the presence of the metal. However, the difference is not significant. During the experiment, we noticed that the MG1655 extract has some condensations, which could affect the results (large variation). Need to redo the experiment using another tube of cell extract.

The graph below is for the ArsR construct fluorescence in CH34 cell extract. Addition of metals have actually made a difference in the fluorescence. The significant difference in fluorescence can be detected at 30 mins.


ArsR downstream construct
Normalised fluorescence
Time/min
0
50
100
150
200
50 ng T7 + 7 uM As (III) [CH34]
50 ng TF construct + 7 uM As (III) [CH34]
50 ng TF construct only [CH34]


At 30 mins
50 ng TF construct only [CH34]
50 ng TF construct + 7 uM As (III) [CH34]
50 ng T7 + 7 uM As (III) [CH34]
50 ng TF construct only [E.coli]
50 ng TF construct + 7 uM As (III) [E.coli]
50 ng T7 + 7 uM As (III) [E.coli]
** (P=0.0025)
0
20
40
60
Normalised fluorescence



No difference observed for the fluorescence of reactions with or without the presence of metal ions. Indeed, the riboswitch dependent termination uses the mechanism of Rho dependent termination, which cannot stop T7 Rpol transcription. Another construct using Anderson promoter has been designed.

11/10/2020 (Nan)

IVT for ArsR construct

Redo the IVT for MG1655 cell extract, adding 50ng DNA template and 7uM As (III)

Sample	Stock conc	Final conc	Blank (ul)	Pos ctl (T7 construct)	TF + 7 uM As	TF only
OTRDB buffer	10X	1X	1.5	1.5	1.5	1.5
DFHBI fluorophore	200 uM	10 uM	0.75	0.75	0.75	0.75
NTP	20 mM	2 mM	1.5	1.5	1.5	1.5

T7 Rpol	50000 U/ml	1.5 U/ul	0.45	0.45	0.45	0.45
Cell extract		30% v/v	4.5	4.5	4.5	4.5
Template	ArsR 1 uM	50 ng		T7: 0.424	0.358	0.358
	100 uM	7 uM	1.05	1.05	1.05	
Nuclease-free water		Fill up to **15** ul	5.25	4.826	4.892	5.942

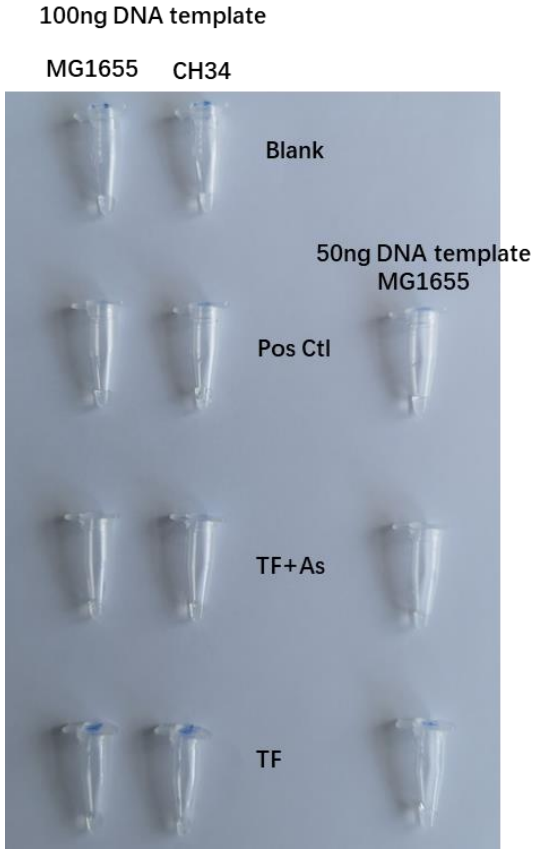
IVT for MG1655/CH34 cell extract, adding 100ng DNA template and 7uM As (III)

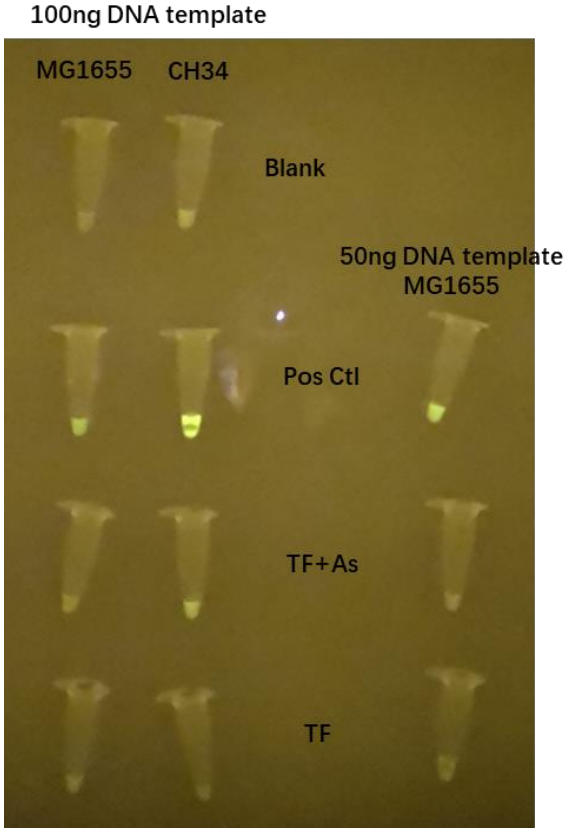
Sample	**Stock conc**	**Final conc**	**Blank (ul)**	**Pos ctl (T7 construct)**	TF + 7 uM As	TF only
OTRDB buffer	10X	1X	1.5	1.5	1.5	1.5
DFHBI fluorophore	200 uM	10 uM	0.75	0.75	0.75	0.75
NTP	20 mM	2 mM	1.5	1.5	1.5	1.5
T7 Rpol	50000 U/ml	1.5 U/ul	0.45	0.45	0.45	0.45
Cell extract		30% v/v	4.5	4.5	4.5	4.5
Template	ArsR 1 uM	100 ng		T7: 0.847	0.716	0.716
	100 uM	7 uM	1.05	1.05	1.05	

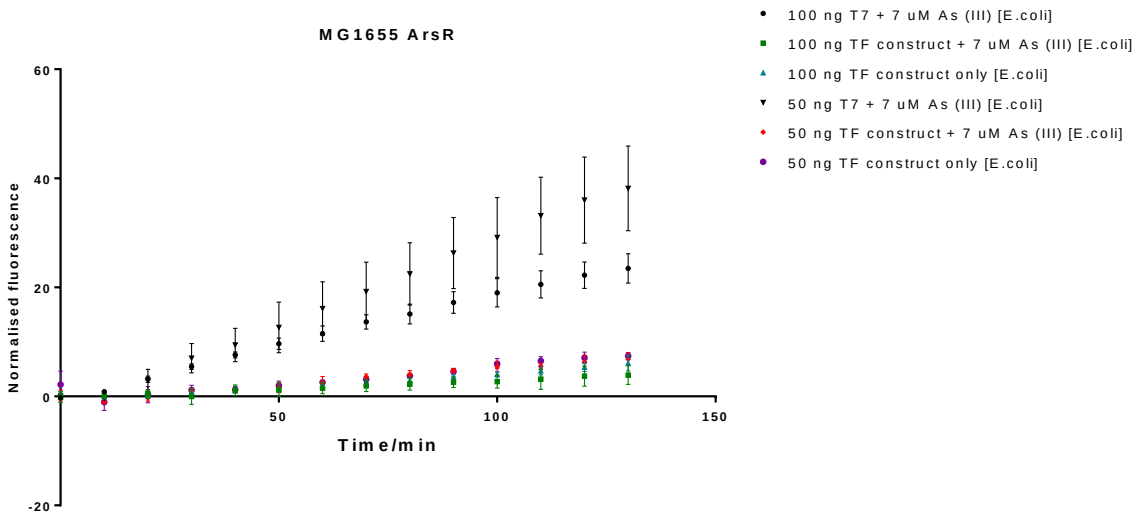
Nuclease-free water		Fill up to **15** ul	5.25	4.403	4.534	5.584

Increasing the DNA template concentration might improve the sensitivity of the system since more TF will become bind to the template and are capable of sensing the metal.

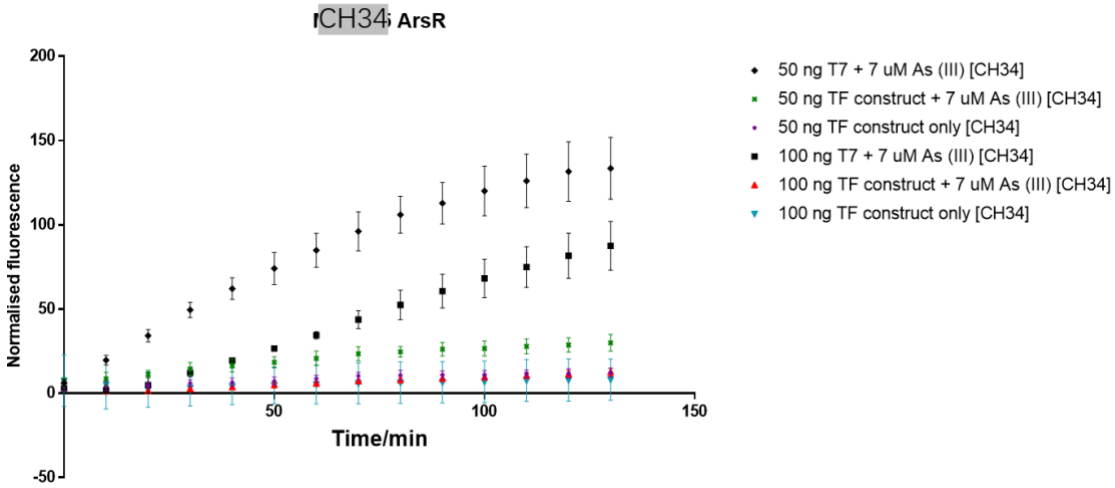
Results:



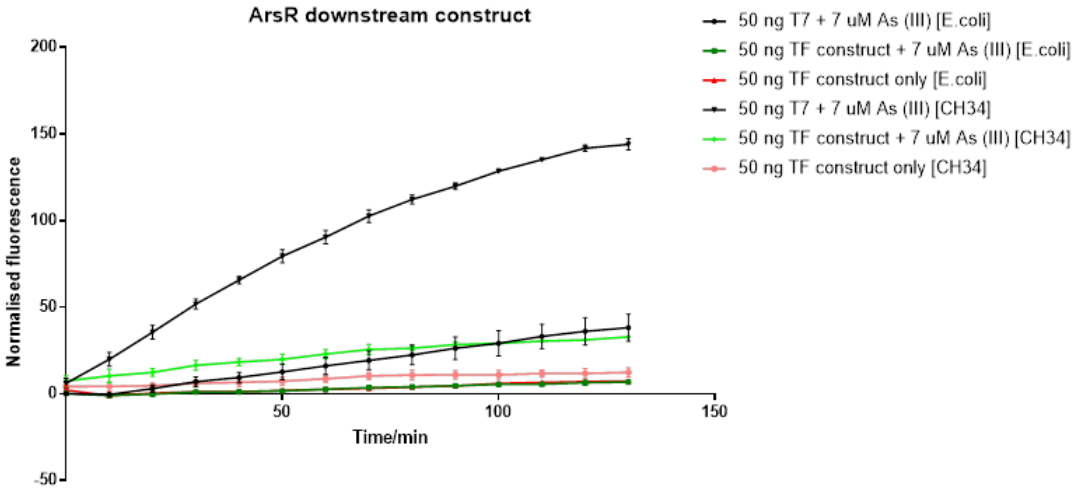




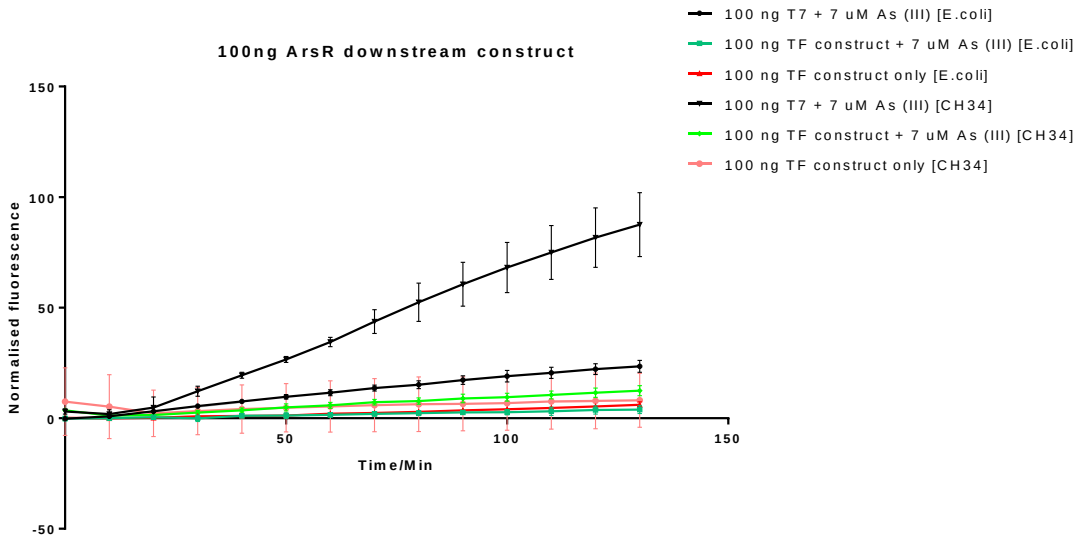
No significant difference can be observed for the 50ng and 100ng DNA template in MG1655 cell extract.



No significant difference can be observed for the 50ng and 100ng DNA template in CH34 extract.



The overall fluorescent intensity in CH34 system is higher than in MG1655. Also, the metal sensing seems to be more sensitive in CH34 system.



T7 construct ligation (making more stock)

	Concentration	Volume
Upstream clip	40 µM	4 µl
Downstream clip	40 µM	4 µl
T4 ligase buffer (+ATP)	10x (10 mM)	1 µl
T4 ligase	400000 U/ml	1 µl

Total volume: 10 µl. Final concentration: 16 µM.

Incubate at 16℃ for 1 h. The remaining 5ul was incubated at 65℃ for 10mins. Which then mixed with 5ul of water to make final concentration of 8uM.

Taken 5ul from each tube, mixed and incubate at 16℃ for 1.5h. 65℃ inactivation for 10 mins. Add 10ml water into the 10ul system.

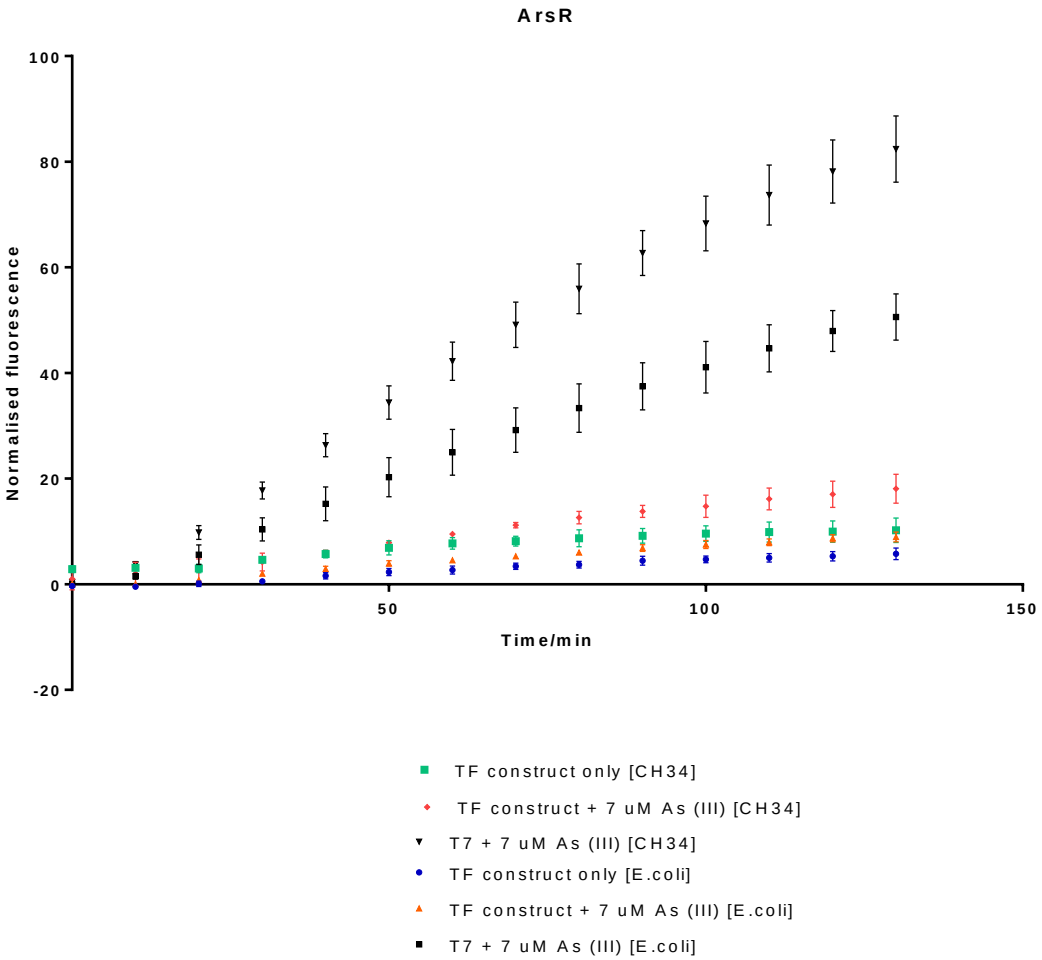
Total Volume: 20 ul. Final concentration: 4uM.

Week 5

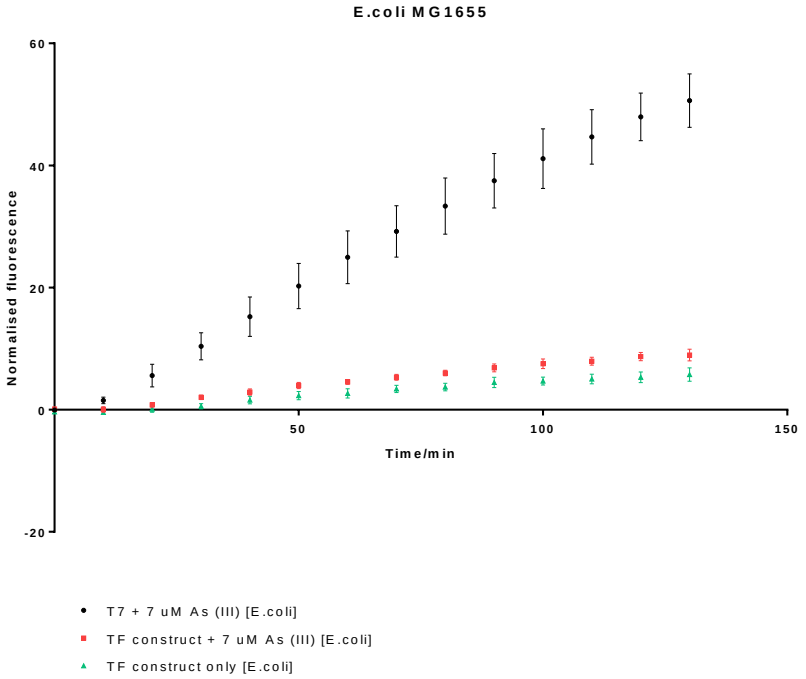
12/10/2020 (Nan)

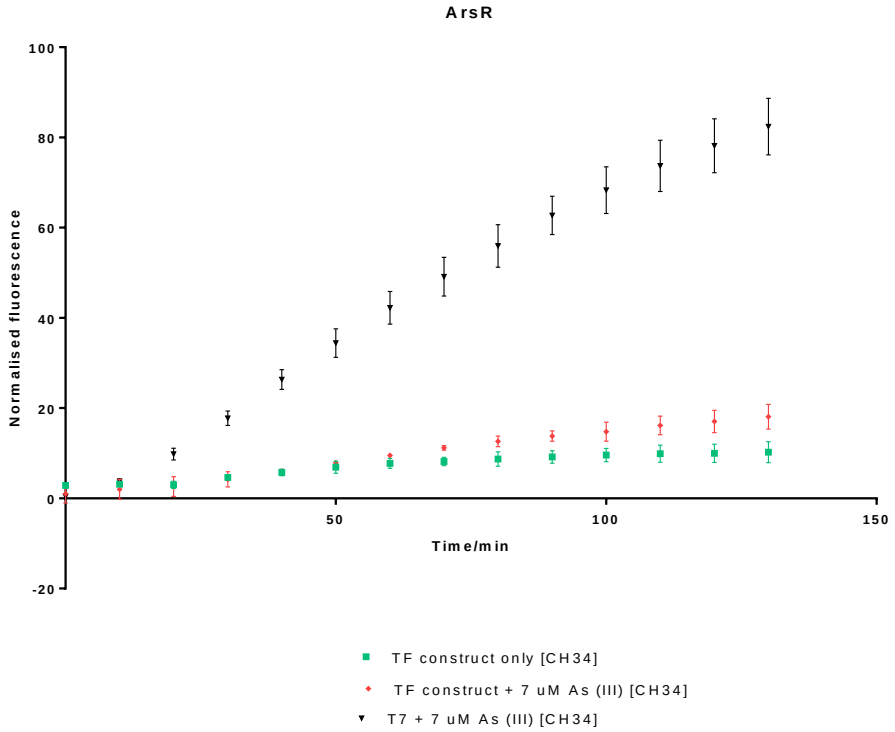
IVT of 100 ng DNA and 0.75ul of T7 RNA polymerase

Sample	Stock conc	Final conc	Blank (µl)	Pos ctl (T7 construct)	TF + 7 µM As	TF only
OTRDB buffer	10X	1X	1.5	1.5	1.5	1.5
DFHBI fluorophore	200 µM	10 µM	0.75	0.75	0.75	0.75
NTP	20 mM	2 mM	1.5	1.5	1.5	1.5
T7 Rpol	50000 U/ml		0.75	0.75	0.75	0.75
Cell extract		30% v/v	4.5	4.5	4.5	4.5
Template	ArsR 1 µM	100 ng		T7: 0.847	0.716	0.716
	100 µM	7 µM	1.05	1.05	1.05	
Nuclease-free water		Fill up to **15** µl	4.95	4.103	4.234	5.284



- Fluorescence for T7 IVT is higher in CH34 cell extract than MG1655, more transcript might be degraded in MG1655.
- ArsR construct has low fluorescence than the T7 construct, although they are using the same promoter. Could suggest the ArsR downstream of the T7 promoter cannot be released efficiently upon metal binding. Might be worth trying the other construct
- The fluorescent difference for the metal adding and no metal system are not significantly different





13/10/2020 (Nan)

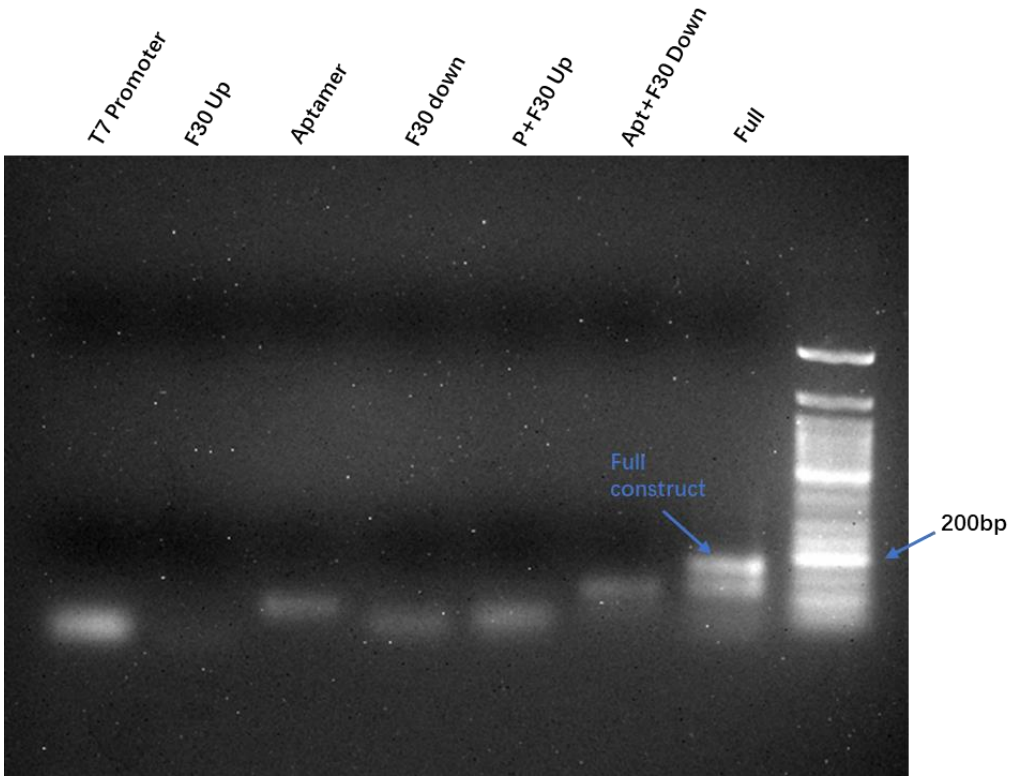
2% agarose gel (T7 construct)

50ml TAE+1g agarose+0.5µl SYBR safe (10000x)

5ul of 50bp DNA ladder

T7 promoter, F30 upstream, aptamer, F30 downstream, T7+F30 up, aptamer+F30 down: conc: 8 uM, 100ng in 5ul loading volume

Full construct: 4 uM, 100ng in 5ul loading volume



15/10/2020

Bubble duplex system:

Bubble 1 forward: GGA TAC TTA CAG CCA TAT CAG TTA CGC CTA CTC CAT TCC ATC CCG GGT TCG TCC AA

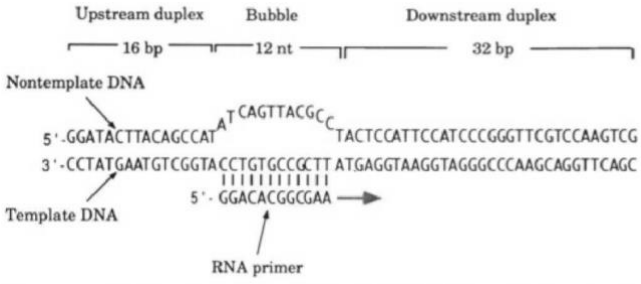
Bubble 1 reverse: GGG ATT GGA CGA ACC CGG GAT GGA ATG GAG TAT TCG CCG TGT CCA TGG CTG TAA GTA TCC

BubblePrimer1:CAT GGA CAC GGC GAA

PrimerGen1_F: GAA ATT AAT ACG ACT CAC TAT AGG GAG ACC ACA ACT CCC GGA CAC GGC GAA

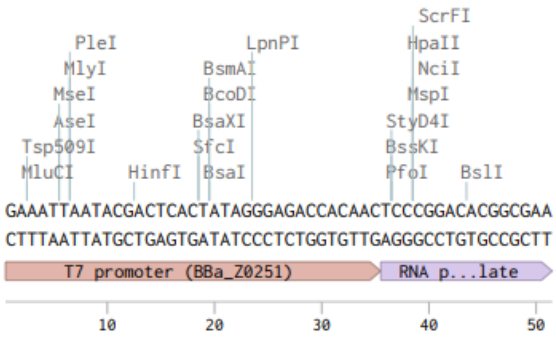
PrimerGen1_R: TTC GCC GTG TCC GGG AGT TGT GGT CTC CCT ATA GTG AGT CGT ATT AAT TTC

Bubble 1 is the bubble duplex that is illustrated below, which consists of a PhytoBrick fusion site, AGGG for ligation with downstream F30 up:iSpinach:F30 dw.



BubblePrimer1 is a DNA primer same as the one marked as RNA primer in the graph, which complements with the bubble region and enables T7 RNA polymerase to extend the transcription from the bubble to the downstream aptamer and produce fluorescence.

PrimerGen1 is just the T7 promoter followed by the RNA primer coding sequence, it is the upstream of the bubble duplex system.



Splint1: CGT CTC AAT GAG ACG CGA CT

This is a short oligo that complement with the reverse aptamer sequence. Ideally, when the reverse aptamer sequence has been ligated into a circle, this short oligo can act as a template for T7 RNA polymerase to perform rolling circle transcription and to achieve signal amplification.

Parts

PrimerGen1 Forward	22.9 nmol = 0.36 mg, Tm=68.4℃
PrimerGen1 Reverse	24.7 nmol = 0.39 mg, Tm=68.4℃
Bubble 1 Forward	12.9 nmol = 0.22 mg, Tm=69.7℃
Bubble 1 Reverse	24.7 nmol = 0.46 mg, Tm=71.7℃
BubblePrimer1 Sequence	15.4 nmol = 0.07 mg, Tm=52.4℃
Splint1	27.9 nmol = 0.17 mg, Tm=57℃

Phosphorylation and annealing

1. Spin the tubes containing the lyophilized oligonucleotides briefly (30 s, 2000 g) to ensure that the pellet is the bottom of the tube.

2. Add nuclease free water to obtain a final concentration of 100 μM. Mix by flicking the tube, and spin briefly (30 s, 2000 g) to ensure that all liquid is at the bottom of the tube. Place the tubes on ice.

3. To phosphorylate oligonucleotides, combine the following in a 0.2 ml clear PCR tube:

[**For PrimerGen1 and Bubble 1**]

Components	Stock conc	Volume (ul)
Forward part	100 uM	10
Reverse part	100 uM	10
T4 PNK buffer (NEB)	10x	2.5
T4 Polynucleotide Kinase	10000 U/ml	0.25
ATP	10 mM	2.5

Volume of the system is 25.25 ul. Concentration of the construct is 40 uM.

[For reverse aptamer, phosphorylation only]

Components	Stock conc	Volume (ul)
Reverse aptamer	100 uM	5
T4 PNK buffer (NEB)	10x	1
T4 Polynucleotide Kinase	10000 U/ml	0.1
ATP	10 mM	1
Nuclease free H_2O		2.9

Volume of the system is 10ul. Concentration is 50 uM.

4. Mix by flicking the tubes, and spin briefly (30 s, 2000 g) to return all liquid to the bottom of the tube. Place the tubes in a heat-block and incubate for 30 min at 37°C (phosphorylation).
5. To anneal, place the tubes with the phosphorylated oligonucleotides in a heat-block at 95°C. Slowly cool down the block from 95°C by turning off the heat-block, and let it cool down to room temperature.

Ligation:

First ligation:

	Concentration	Volume
Bubble 1	40µM	2 µl
F30 up	40µM	2 µl
T4 ligase buffer (+ATP)	10x (10mM)	1µl
T4 ligase	400000 U/ml	0.5µl
Nuclease free H2O		4.5

Final volume: 10 ul. Final concentration 8 uM.

4. Mix by flicking the tubes, and spin briefly (30 s, 2000 g) to return all liquid to the bottom of the tube.
5. PCR machine was set to incubate at 16°C for 1 hours

Second ligation:

	Concentration	Volume
Bubble 1 + F30 up	8µM	5 µl
Aptamer + F30 down	8µM	5 µl
T4 ligase		0.25µl

Final volume: 10.25 ul. Final conc: 4 uM

1. The remaining 5ul need to be incubated at 65°C 10 mins for enzyme inactivation
2. PCR machine was set to incubate at 16°C for 2 hours, which is followed by incubation at 65°C for 10 mins

Aptamer reverse ligation

	Concentration	Volume
Aptamer reverse	50μM	2 μl
T4 ligase buffer (+ATP)	10x (10mM)	1μl
T4 ligase	400000 U/ml	0.5μl
Nuclease free H2O		6.5

Final volume: 10 ul. Final conc: 10 uM

2% agarose gel: 50ml TAE+1g agarose+0.5μl SYBR safe (10000x)

50bp ladder: 2.5 ul;

Sample:

Bubble 1: 100 ng/lane

F30 up: 100 ng/lane

Aptamer: 100 ng/lane

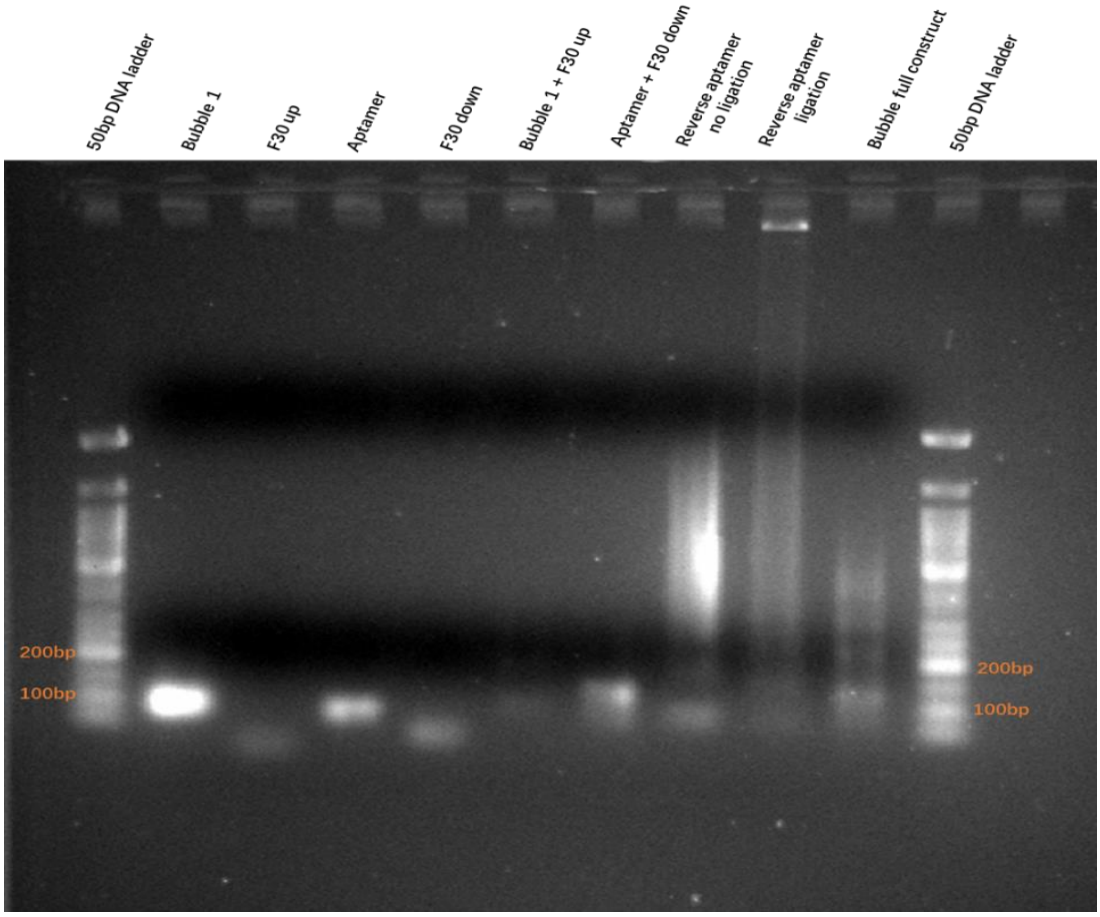
F30 down: 100 ng/lane

Bubble 1+F30 up: 100 ng/lane

Aptamer+F30 down: 100 ng/lane

Reverse aptamer without ligation: 100 ng/lane

Reverse aptamer with ligation: 100 ng/lane



For the bubble duplex construct. The half construct seems to be ligating ok, but the resolution for the band is quite low. The bubble full construct has multiple bands at ~100bp, 200bp, 300bp, and even at ~500bp, but they all quite fainted.

Also not sure about whether the reverse aptamer ligation worked or not from the gel.

16/10/2020

Target sequence the Stanford team wants to detect:

GTCCTTTGCTCGGAAGAGTATGAAGATGAACAAAGC

Stanford toehold construct

Toehold forward: tccc GCTTT GTTCA TCTTC ATACT CTTCC GAGCA AAGGA CGGAA

ATGTA GGAGG AAAGA CACAT TATGG TCCTT TGCTC AAACC TGGCG GCAGC GCA

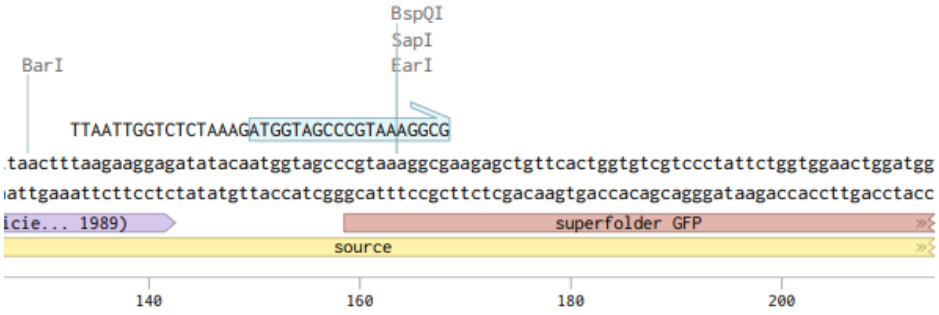
Toehold reverse: CTTT TGC GCTGC CGCCA GGTTT GAGCA AAGGA CCATA ATGTG

TCTTT CCTCC TACAT TTCCG TCCTT TGCTC GGAAG AGTAT GAAGA TGAAC AAAGC

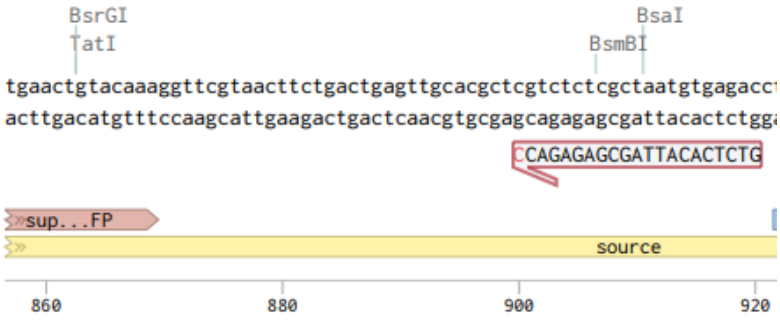
Reporter forward: 5’ TTAATTGGTCTCTAAAGATGGTAGCCCGTAAAGGCG 3’

Reporter reverse: 5’ GTCTCACATTAGCGAGAGACC 3’

Forward primer matching to the pJUMP-48-2A(sfGFP) plasmid

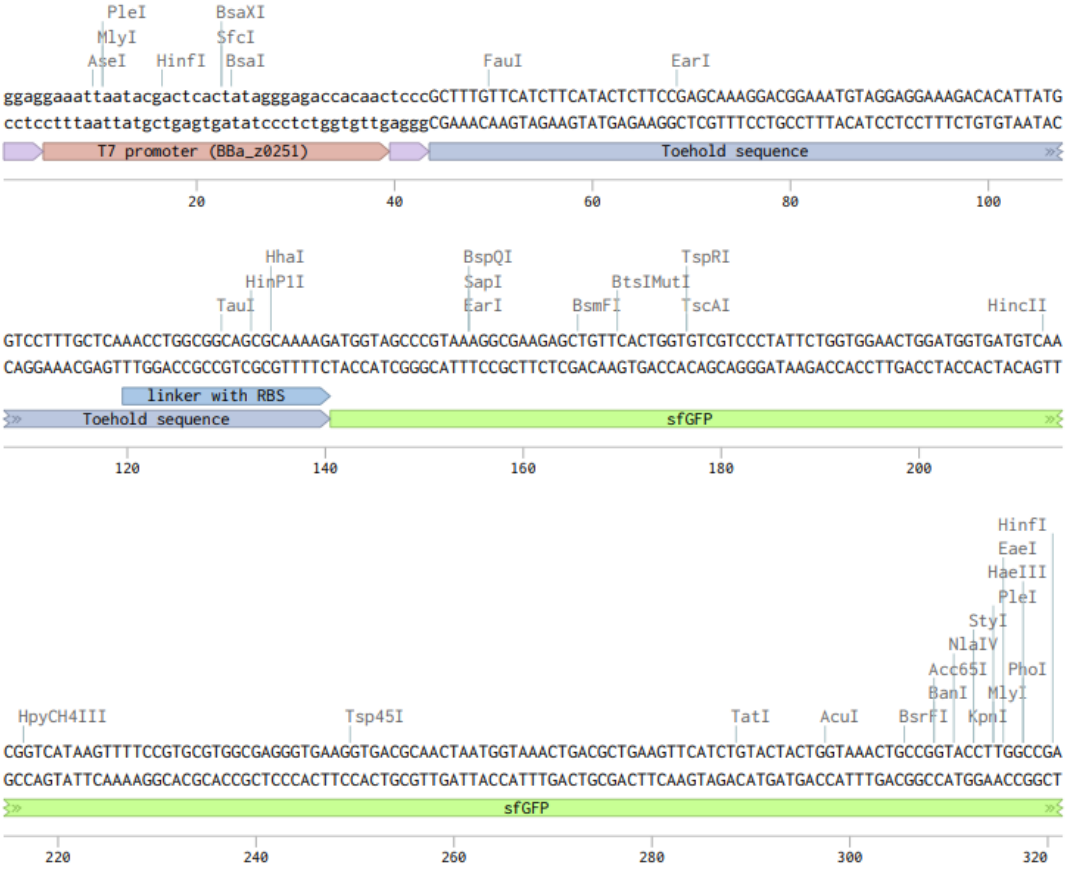


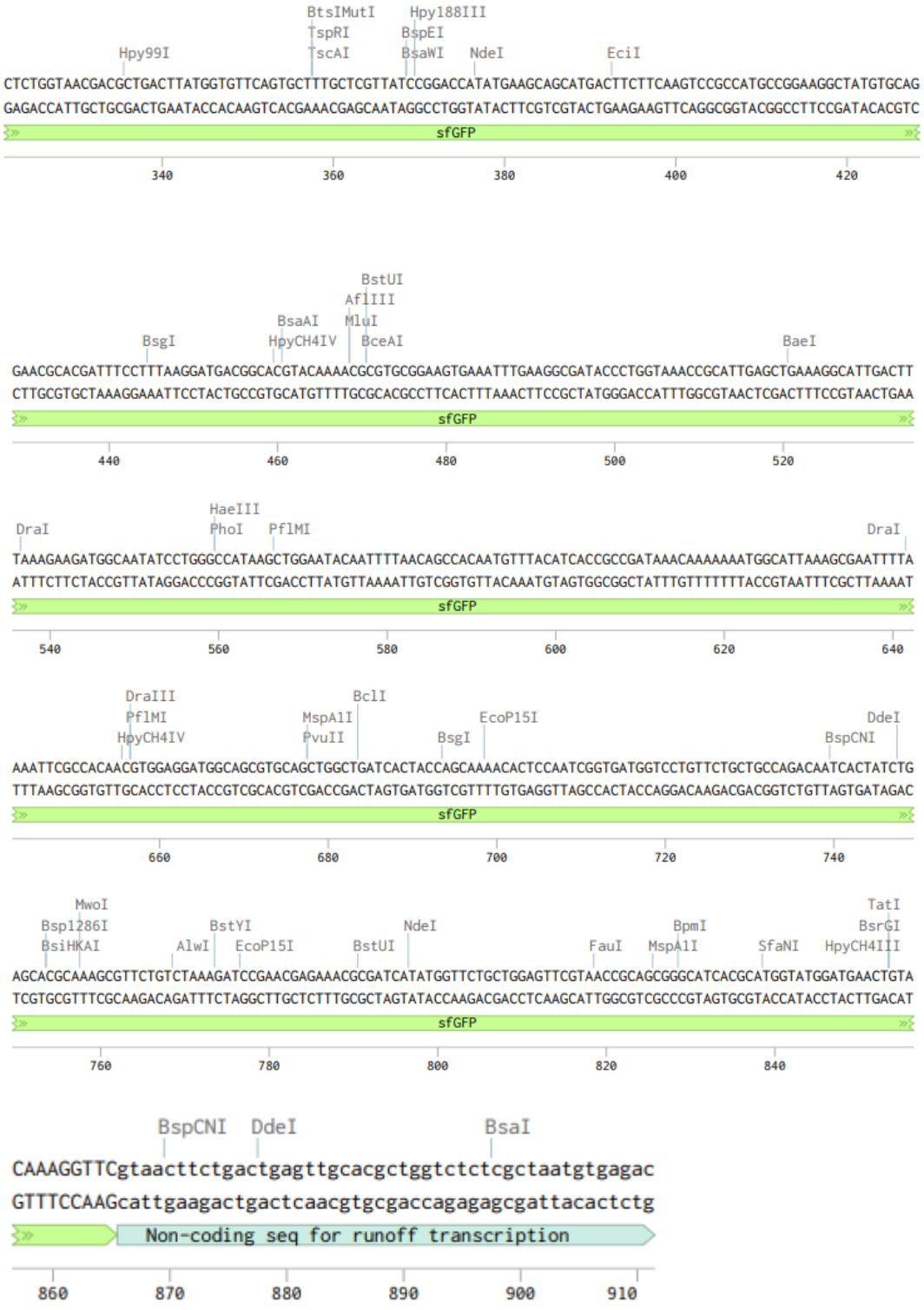
Reverse primer matching to the pJUMP-48-2A(sfGFP) plasmid (with one mismatch)



Complete toehold construct

Toehold stanford (911 bp)





Parts

StanfordTarget1	

ToeholdF1	115.9 nmol = 3.46 mg, Tm=72.9℃
ToeholdR1	94.6 nmol = 2.82 mg, Tm=71.8℃
ReporterF1	21.8 nmol = 0.24 mg, Tm=63.1℃
ReporterR1	27 nmol = 0.17 mg, Tm=54.7℃
ProFluoroF1	20.2 nmol = 0.38 mg, Tm=68.2℃
ProFluoroF2	22.3 nmol = 0.41 mg, Tm=66.2℃
ProFluoroR1	18.4 nmol = 0.34 mg, Tm=67℃
ProFluoroR2	23.9 nmol = 0.44 mg, Tm=67.1℃

Phosphorylation and annealing

1. Spin the tubes containing the lyophilized oligonucleotides briefly (30 s, 2000 g) to ensure that the pellet is at the bottom of the tube.
2. Add nuclease free water to obtain a final concentration of 100 μM. Mix by flicking the tube, and spin briefly (30 s, 2000 g) to ensure that all liquid is at the bottom of the tube. Place the tubes on ice.
3. To phosphorylate oligonucleotides, combine the following in a 0.2 ml clear PCR tube:

   [3 reactions: Toehold, proFluoro upstream, proFluoro downstream]

Components	Stock conc	Volume (ul)
Forward part	100 uM	10
Reverse part	100 uM	10
T4 PNK buffer (NEB)	10x	2.5

T4 Polynucleotide Kinase	10000 U/ml	0.25
ATP	10 mM	2.5

Volume of the system is 25.25 ul. Concentration of the construct is 40 uM.

4. Mix by flicking the tubes, and spin briefly (30 s, 2000 g) to return all liquid to the bottom of the tube. Place the tubes in a heat-block and incubate for 30 min at 37°C.
5. To anneal, place the tubes with the phosphorylated oligonucleotides in a heat-block at 95°C. Slowly cool down the block from 95°C by turning off the heat-block, and let it cool down to room temperature.

PCR of sfGFP

Single bacteria colony with transformed pJUMP-48-2A(sfGFP) plasmid containing sfGFP sequence was picked and suspended in 200 ul H2O.

Phusion DNA polymerase

	Concentration	Volume/µl
Forward primer	10µM	1
Reverse primer	10µM	1
Cell template		1
Phusion High Fidelity Master Mix buffer	2x	12.5
Nuclease-free H2O		9.5
Total volume		25

Temperature	Time	
94℃ denaturation	5 minutes	
94℃ denaturation	30 seconds	35 cycle
67℃ annealing	30 seconds	
72℃ extension	15 sec/kb	
72℃ final extension	3 minutes	

*The annealing temperature was recommended be the NEB website, Tm calculator.

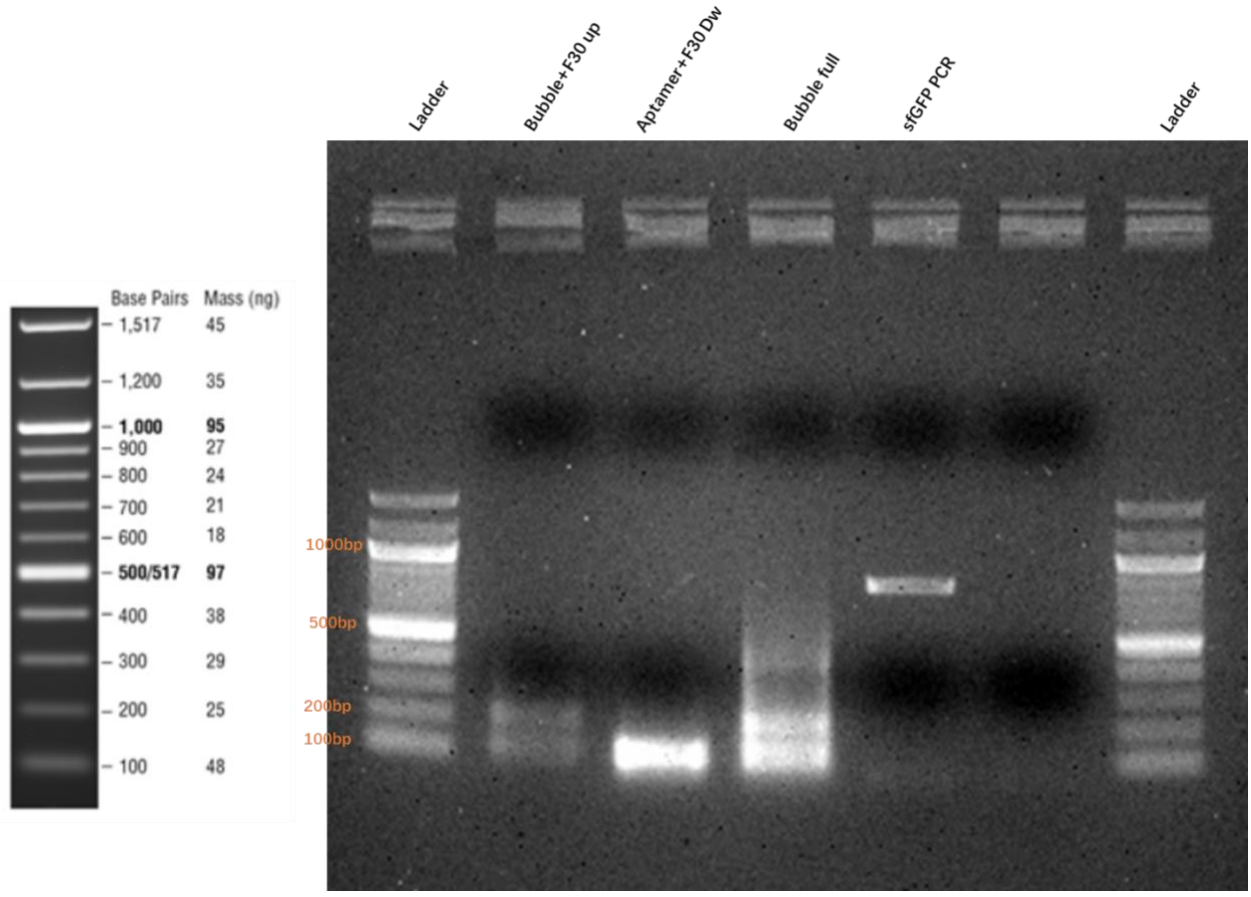
16/10/2020

IVT

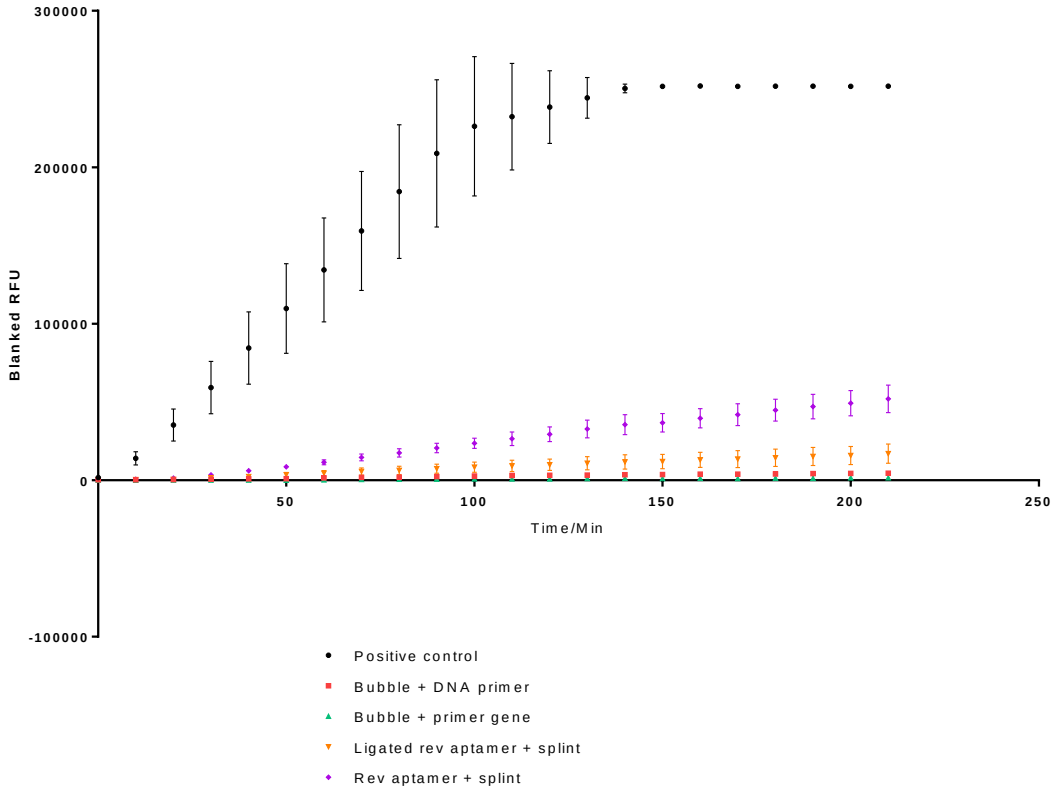
	Stock conc	**Final conc**	**Negative control (No construct)**	**Positive control (T7 construct)**	**Exp 1 (Bubble 1 + DNA primer)**	**Exp 2 (Bubble 1 + primer gene 1)**	**Exp 3 (ligated reverse apt + Splint1)**	**Exp 4 (reverse apt + Splint1)**	**Reaction mix**
	C	C	V	V	V	V	V	V	V
OTRDB	10x	1x	2µl	2µl	2µl	2µl	2µl	2µl	40
DFHBI Fluorophore	200µM	10µM	1µl	1µl	1µl	1µl	1µl	1µl	20
NTPs	20mM	2mM	2µl	2µl	2µl	2µl	2µl	2µl	40
T7 RNAP			0.75µl	0.75µl	0.75µl	0.75µl	0.75µl	0.75µl	
T7 construct	4 µM	100ng		0.212µl					
Bubble 1	4 µM	100ng			0.194µl	0.194µl			
DNA primer	100 µM	100ng			0.217µl				
Primer gene 1	40 µM	100ng				0.1µl			

Ligated reverse aptamer	10 µM	100ng					0.381 µl		
Reverse aptamer	50 µM	100ng						0.1 µl	
Splint1	100 µM	100ng					0.164 µl	0.164 µl	
Nuclease free H_2O		Final volume: 20ul	14.25 µl	14.038 µl	13.839 µl	13.956 µl	13.705	13.986	

1.5% agarose gel (100bp ladder), 35mins



Didn't run the bubble alone, so it is hard to interpret the two bands with exactly half the size of each other for the bubble+F30 up construct. But there seems to have a band at ~500bp for the complete bubble construct. The PCR product for the sfGFP seems to have correct length (~800bp).



The linear reverse aptamer sequence (purple) gave some fluorescence, which indicates the T7 RNA polymerase can use splint as a primer for transcription. The ligated reverse aptamer gave less fluorescence (orange); however, I suspect the emitted fluorescence could be the result of the unsuccessfully ligated linear reverse aptamer. The bubble construct did not work.

18/10/2020

T7+Toehold ligation

	Concentration	Volume
T7	40µM	2 µl
Toehold	40µM	2 µl
T4 ligase buffer	10x	1µl

(+ATP)	(10mM)	
T4 ligase	400000 U/ml	0.5µl
Nuclease free H2O		4.5

Final volume: 10 ul. Final concentration 8 uM.

Ribo upstream, downstream and F30 up ligation

	Concentration	Volume
Ribo upstream	40µM	2 µl
Ribo downstream	40µM	2 µl
F30 up	40µM	2 µl
T4 ligase buffer (+ATP)	10x (10mM)	1µl
T4 ligase	400000 U/ml	0.5µl
Nuclease free H2O		2.5

Final volume: 10 ul. Final concentration 8 uM.

- Mix by flicking the tubes, and spin briefly (30 s, 2000 g) to return all liquid to the bottom of the tube.
- PCR machine was set to incubate at 16°C for 1.5 hours

sfGFP PCR product purification using Qiagen minipret.

1. All the PCR product (25ul) was added onto the column. Wash the QIAprep spin column by adding 0.5 ml Buffer PB. Centrifuge for 30–60 s and discard the flow-through.
2. Wash the QIAprep spin column by adding 0.75 ml Buffer PE. Centrifuge for 30–60 s and discard the flow-through
3. Transfer the QIAprep spin column to the collection tube.
4. Centrifuge for 1 min to remove residual wash buffer.
5. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 30 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of the QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.

Nanodrop using EB to blank and the concentration measured is 42.3 ng/ul in 50ul EB. So in 30ul EB, the concentration is 70.5 ng/ul.

Bsal-HF V2 digestion and inactivation

Sample	Stock conc	Volume/ul
sfGFP PCR product	70.5 ng/ul	7.5
Cutsmart buffer	10x	5
Bsal-HF V2		0.5 (10 units)
H2O		37
Final volume		50

The reaction system was incubated at 37℃ for 15mins, which was followed by inactivation at 65℃ for 20 mins.

Ligation of:

Stanford toehold full construct

	Stock Concentration	Volume
T7+toehold	8µM	2.5 µl
sfGFP	10.575 ng/ul	25 µl
T4 ligase buffer (+ATP)	10x (10mM)	2.5 µl
T4 ligase	400000 U/ml	0.5µl

Anderson promoter_ F riboswitch full construct

	Stock Concentration	Volume
F Ribo up dw F30 up	8µM	5 µl
aptamer F30 dw	8µM	5 µl
T4 ligase	400000 U/ml	0.25µl

Incubated at 16℃ for 2hs, 65℃ inactivation for 10 mins, 4℃ storage overnight.

Week 6

19/10/2020

Since the previous agarose gels have bad resolution, I changed the buffer from TAE to TBE,

which can resolve bands with small molecular weight better.

-Making 500ml 5x TBE

Tris base	27 g

Boric acid	13.75 g
EDTA	2.34 g
Adjust pH to 8.3 with NaOH	

-PCR of the ligated full toehold

Phusion DNA polymerase

	Stock Concentration	Volume/μl
Forward primer	10μM	1
Reverse primer	10μM	1
Cell template	8.66 ng/ul	1
Phusion High Fidelity Master Mix buffer	2x	12.5
Nuclease-free H2O		9.5
Total volume		25

Temperature	Time	
98℃ denaturation	5 minutes	
98℃ denaturation	30 seconds	35 cycle
62℃ annealing	30 seconds	
72℃ extension	15 sec/kb	
72℃ final extension	5 minutes	

-Run the gel (Riboswitch full construct and Toehold full construct)

2% agarose with 50ml TBE + 1g agarose + 0.5ul CYBR safe

100bp ladder

Ribo up: 100ng, 40uM, 0.1S + 1 dye + 3.9 H2O

Ribo dw: 100ng, 40uM, 0.1S + 1 dye + 3.9 H2O

Ribo + F30 up: 100ng, 8uM, 0.14S + 1 dye + 3.86 H20

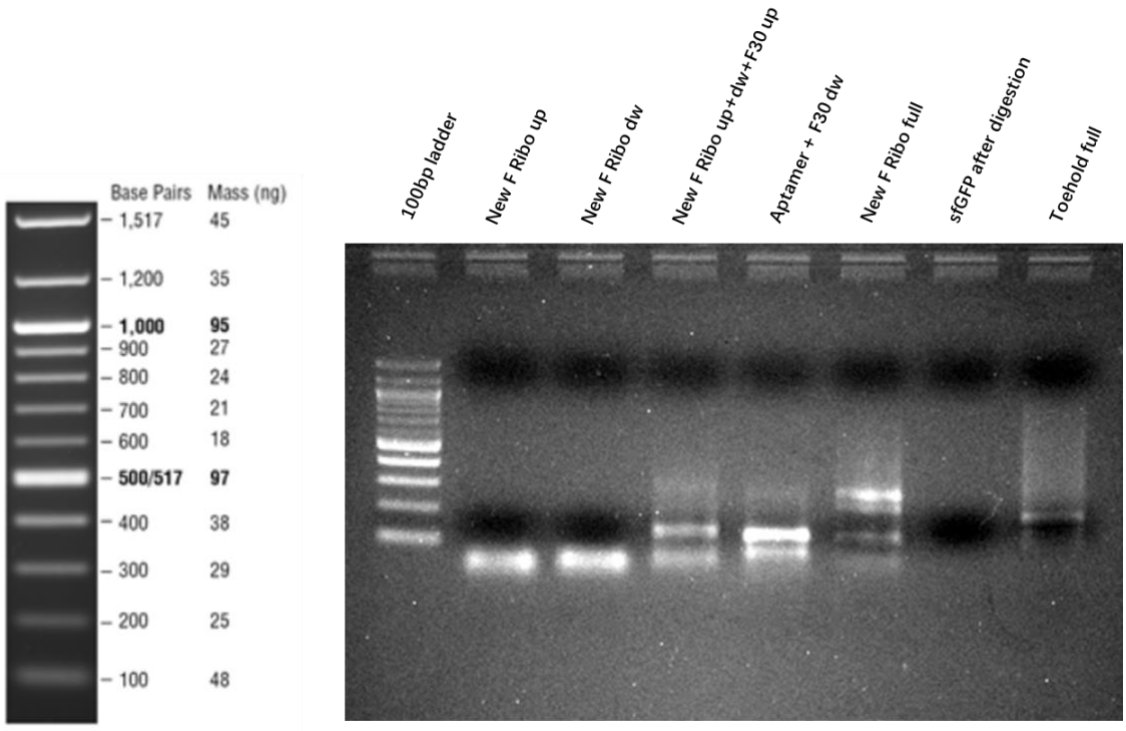
Aptamer + F30 dw: 100ng, 16uM, 0.156S + 1 dye + 3.844 H2O

Ribo full: 100ng, 4uM, 0.15S + 1 dye + 3.85 H2O

sfGFP: 1s + 1 dye + 3 H2O

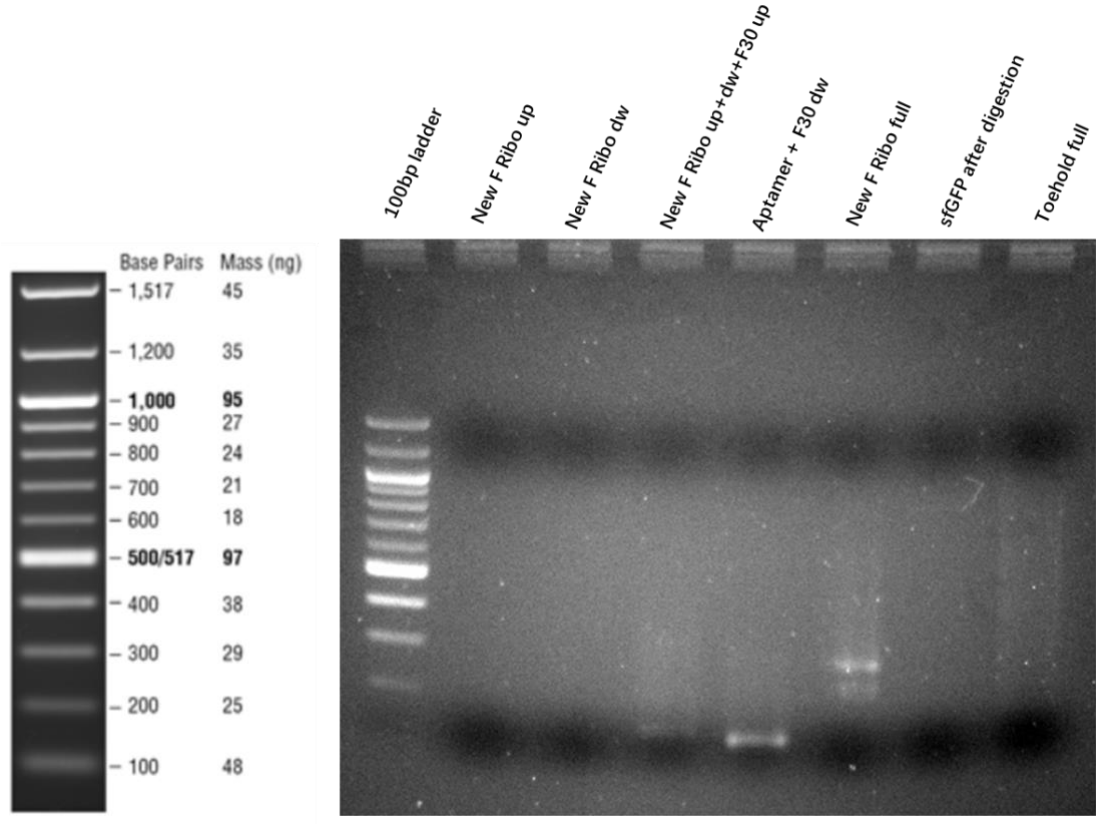
Toehold full: 1s + 1 dye + 3 H2O

90V for 30 mins and 50 mins



30mins

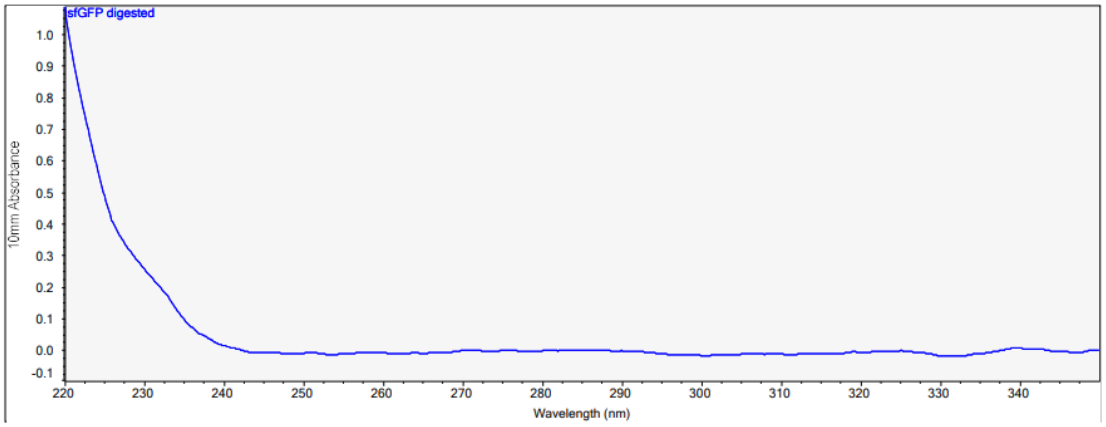
At 30 mins, not all bands are at their final locations. Some close positioned bands might represent same species but in the progress of migration. Ligation of the new riboswitch worked. But not the toehold construct. The gel indicates there are no DNA in the sample after restriction enzyme digestion (forgot to add DNA?), thus, the ligation of the toehold whole construct has failed. Redo the restriction enzyme digestion of purified sfGFP.



50 mins (This time is too long, most samples are run out from the gel.)

DNA concentration measurement for the digested sfGFP

#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
1	sfGFP digested	LI Mengxi	19/10/2020 16:10:36	-0.7	ng/µl	-0.015	-0.009	1.74	-0.06	DNA	50.00



The DNA concentration measurement also agrees with the result shown by gel.

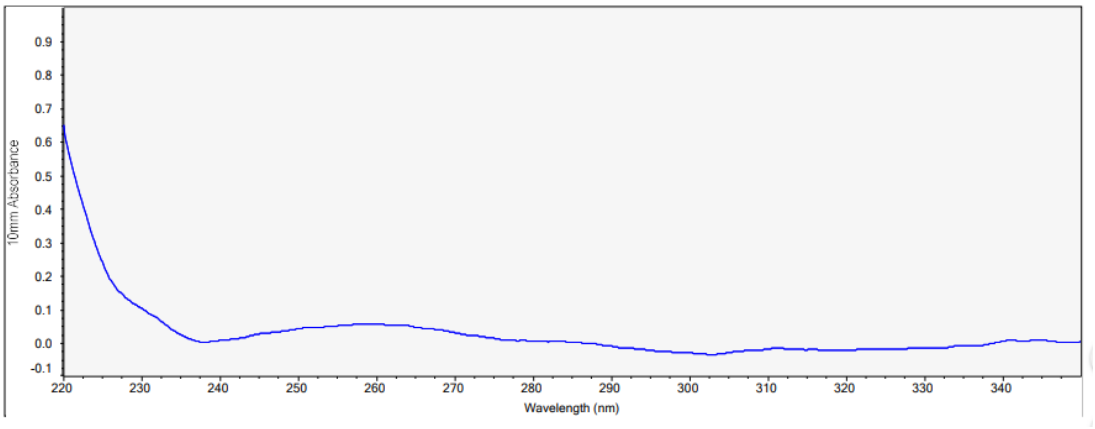
Bsal-HF V2 digestion and inactivation

Sample	Stock conc	Volume/ul
sfGFP PCR product	70.5 ng/ul	7.5
Cutsmart buffer	10x	5
Bsal-HF V2		0.5 (10 units)
H2O		37
Final volume		50

The reaction system was incubated at 37℃ for 15mins, which was followed by inactivation at 80℃ for 20 mins.

DNA concentration of the newly digested sample was measured.

#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
1		LI Mengxi	19/10/2020 17:22:43	2.5	ng/µl	0.051	-0.001	-67.91	0.51	DNA	50.00



According to calculation, the total amount of DNA added into the digestion system is 528ng. The concentration in 50 ul system should be 10.5 ng/ul. Which is 5 times higher than the measured concentration. Could be the problem with the blank (EB was used as blank), or DNA degradation happens.

20/10/2020

-Ligation of T7-toehold with newly digested sfGFP.

	Concentration	Volume/µl
T7-toehold	8µM	2.5
Digested sfGFP		25
T4 ligase buffer	10x	2.5
T4 ligase		0.5

Incubate at 16℃ for 2hs. Inactivation at 65℃ for 10 mins.

-PCR of the toehold whole construct

Phusion DNA polymerase

	Concentration	Volume/µl
T7 Forward primer	10µM	1
sfGFP Reverse primer	10µM	1
Cell template		1
Phusion High Fidelity Master Mix buffer	2x	12.5
Nuclease-free H2O		9.5
Total volume		25

Temperature	Time	
98℃ denaturation	5 minutes	
98℃ denaturation	30 seconds	35 cycle
62℃ annealing	30 seconds	
72℃ extension	15 sec/kb	
72℃ final extension	5 minutes	

2% agarose with 50ml TBE + 1g agarose + 0.5ul CYBR safe

100bp ladder: 2.5 µl

T7+Toehold: 8µM, 100ng, 0.153 µl sample + 1 Dye + 3.847 H2O

Digested sfGFP: 1 µl sample + 1 Dye + 3 H2O

Toehold full construct after PCR: 1 µl sample + 1 Dye + 3 H2O

Bubble 1: 8µM, 100ng, 0.2 µl sample + 1 Dye + 3.8 H2O

F30 up: 8µM, 100ng, 0.776 µl sample + 1 Dye + 3.224 H2O

Aptamer: 8µM, 100ng, 0.235 µl sample + 1 Dye + 3.765 H2O

F30 down: 8µM, 100ng, 0.421 µl sample + 1 Dye + 3.579 H2O

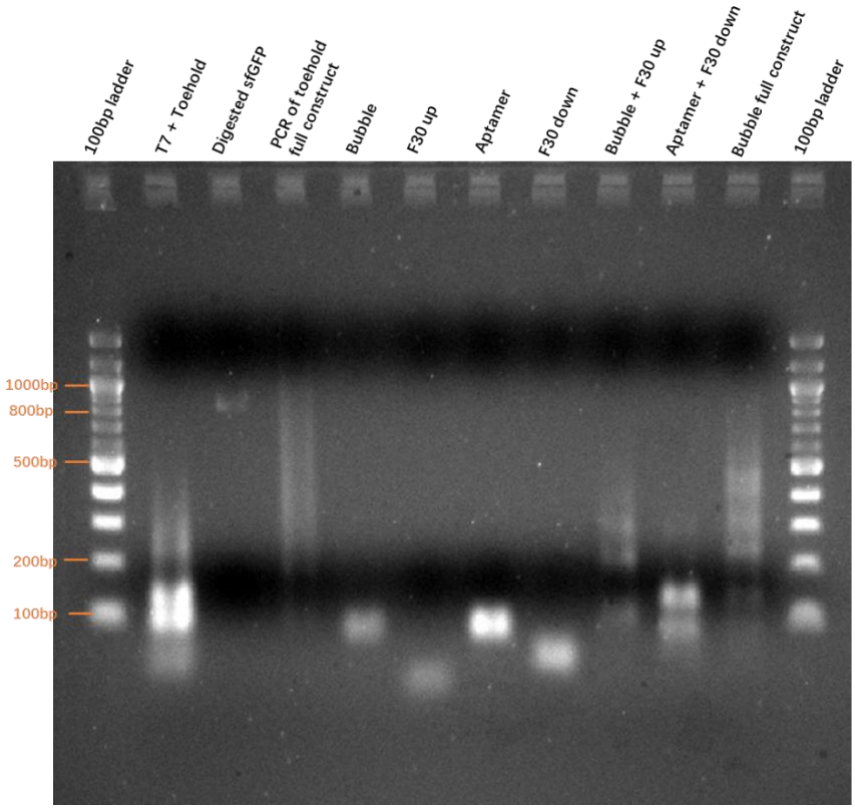
Bubble + F30 up: 8µM, 100ng, 0.247 µl sample + 1 Dye + 3.753 H2O

Aptamer + F30 down: 8µM, 100ng, 0.156 µl sample + 1 Dye + 3.84 H2O

Full bubble construct: 4µM, 100ng, 0.25 µl sample + 1 Dye + 3.75 H2O

100bp ladder: 2.5 µl

90V for 35 mins

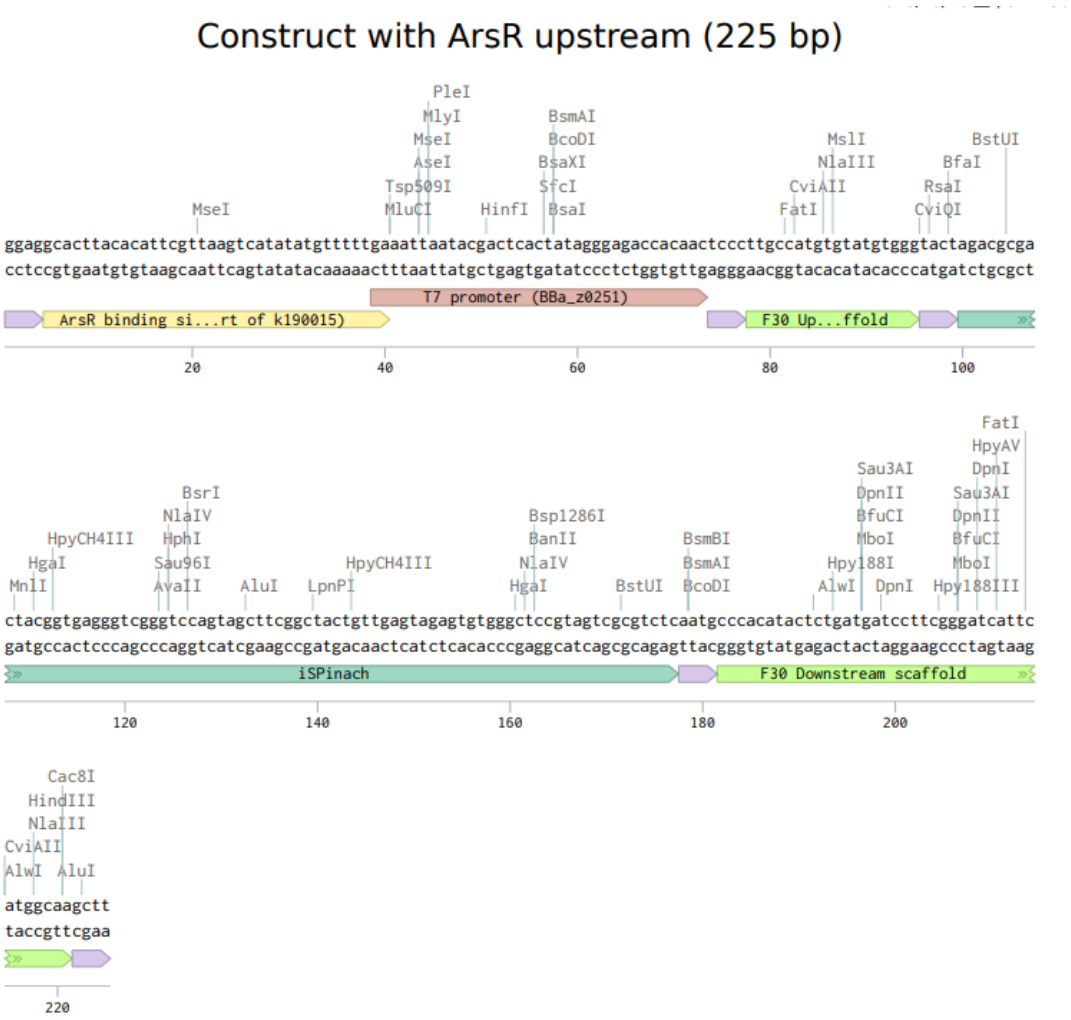


The digested sfGFP seems to have a band at correct molecular weight. However, only a smear appeared in the PCR product.

For the bubble duplex, multiple bands still appear in the bubble ligated construct. Thus, I speculate that the unpaired region in the bubble might be pairing up with other DNA parts overhangs during

ligation to obtain various complexes with distinct sizes. Ligation protocol need to be changed for bubble construction in the future.

21/10/2020



Parts

ArsR_T7 forward	55.8 nmol = 1.26 mg, Tm=66.7℃
ArsR_T7 reverse	71.7 nmol = 1.62 mg, Tm=66.7℃

Phosphorylation and annealing

1. Spin the tubes containing the lyophilized oligonucleotides briefly (30 s, 2000 g) to ensure that the pellet is at the bottom of the tube.
2. Add nuclease free water to obtain a final concentration of 100 µM. Mix by flicking the tube, and spin briefly (30 s, 2000 g) to ensure that all liquid is at the bottom of the tube. Place the tubes on ice.
3. To phosphorylate oligonucleotides, combine the following in a 0.2 ml clear PCR tube:

Components	Stock conc	Volume (ul)
ArsR_T7 forward	100 uM	10
ArsR_T7 reverse	100 uM	10
T4 PNK buffer (NEB)	10x	2.5
T4 Polynucleotide Kinase	10000 U/ml	0.25
ATP	10 mM	2.5

Volume of the system is 25.25 ul. Concentration of the construct is 40 uM.

4. Mix by flicking the tubes, and spin briefly (30 s, 2000 g) to return all liquid to the bottom of the tube. Place the tubes in a heat-block and incubate for 30 min at 37°C.

To anneal, place the tubes with the phosphorylated oligonucleotides in a heat-block at 95°C. Slowly cool down the block from 95°C by turning off the heat-block, and let it cool down to room temperature.

2% agarose with 50ml TBE + 1g agarose + 0.5ul CYBE safe

100bp ladder: 2.5 µl

T7: 40µM, 100ng, 0.115 µl sample + 1 Dye + 3.885 H2O

Toehold: 40µM, 100ng, 0.1 µl sample + 1 Dye + 3.9 H2O

T7+Toehold: 8µM, 100ng, 0.14 µl sample + 1 Dye + 3.86 H2O

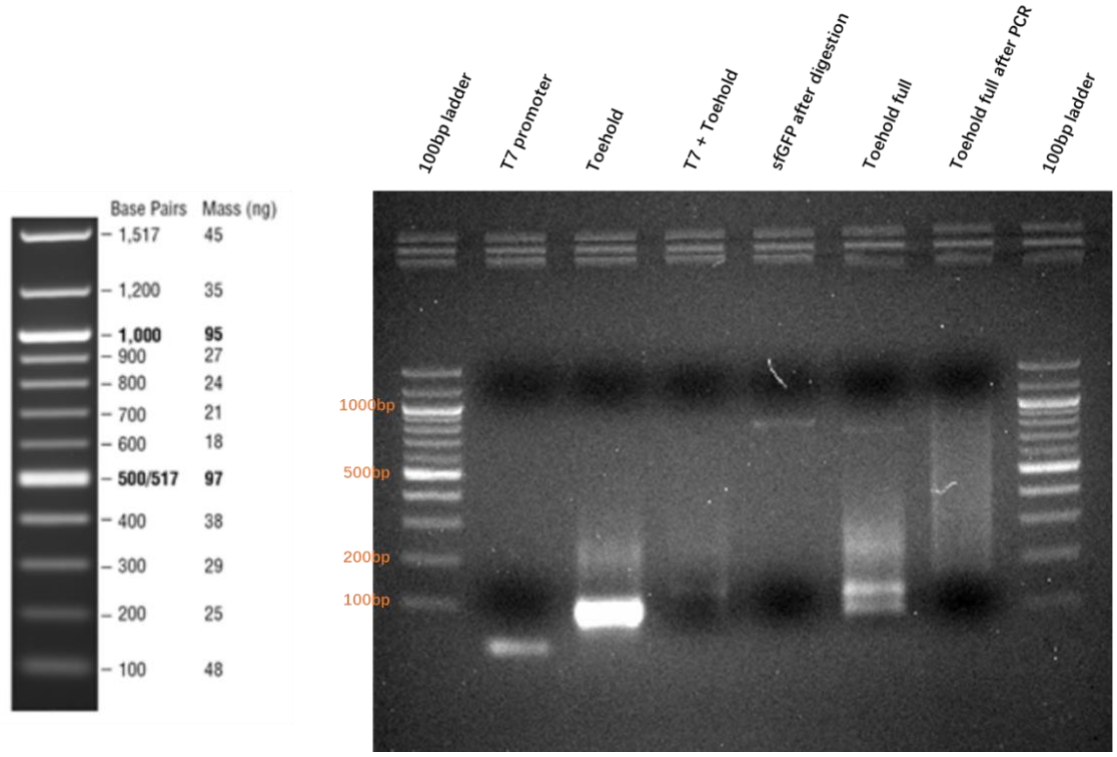
Digested sfGFP: 2 µl sample + 1 Dye + 2 H2O

Ligated Toehold: 2 µl sample + 1 Dye + 2 H2O

Toehold full construct after PCR: 2 µl sample + 1 Dye + 2 H2O

100bp ladder: 2.5 µl

90V for 35 mins



T7+ Toehold seems to be ligating Ok, but the ligated toehold has slightly lower molecular weight?

Maybe the ligation didn’t work.

22/10/2020

Full toehold construct PCR

T7 promoter forward primer:

5’ GGAGGAAATTAATACGACTCACTATAGGGAGACCACAAC 3’

sfGFP reverse primer:

5’ GTCTCACATTAGCGAGAGACC 3’

New ArsR TF construct ligation:

	Concentration	Volume
ArsR promoter	40 µM	4 µl
F30 up	40 µM	4 µl
T4 ligase buffer (+ATP)	10x (10 mM)	1 µl
T4 ligase	400000 U/ml	1 µl

Total volume: 10 µl. Final concentration: 16 µM.

Incubate at 16℃ for 1 h. Add 10ul water into the 10ul system to dilute the system to 8 uM. Taken 5ul from each tube, mixed and incubate at 16℃ for 1.5h. 65℃ inactivation for 10 mins.

Total Volume: 10 ul. Final concentration: 4uM.

The remaining 15ul was incubated at 65℃ 10mins for ligase inactivation.

-PCR of the toehold whole construct

Phusion DNA polymerase

	Stock concentration	Volume/ µl	Volume/ µl

T7 Forward primer	10μM	0.25	0.5
sfGFP Reverse primer	10μM	0.25	0.5
Cell template		1	1
Phusion High Fidelity Master Mix buffer	2x	12.5	12.5
Nuclease-free H2O		11	10.5
Total volume		25	25

Temperature	Time	
98℃ denaturation	5 minutes	
98℃ denaturation	30 seconds	35 cycle
62℃ annealing	30 seconds	
72℃ extension	15 sec/kb	
72℃ final extension	5 minutes	

2% Agarose gel with 50ml TBE + 1g agarose + 0.5ul cyber safe

100bp ladder

ArsR promoter: 8uM (0.263ul Sample + 1 Dye + 3.737 H2O)

F30 up: 8uM (0.776ul Sample + 1 Dye + 3.224 H2O)

Aptamer: 8uM (0.235ul Sample + 1 Dye + 3.765 H2O)

F30 down: 8uM (0.421ul Sample + 1 Dye + 3.579 H2O)

ArsR+ F30 up: 8uM (0.204ul Sample + 1 Dye + 3.796 H2O)

Aptamer + F30 dw: 8uM (0.156ul Sample + 1 Dye + 3.84 H2O)

ArsR full: 4uM (0.18 ul Sample + 1 Dye + 3.82 H2O)

Digested sfGFP (2ul Sample + 1 Dye + 2 H2O)

Ligated sfGFP (2ul Sample + 1 Dye + 2 H2O)

Ligated Toehold full (2ul Sample + 1 Dye + 2 H2O)

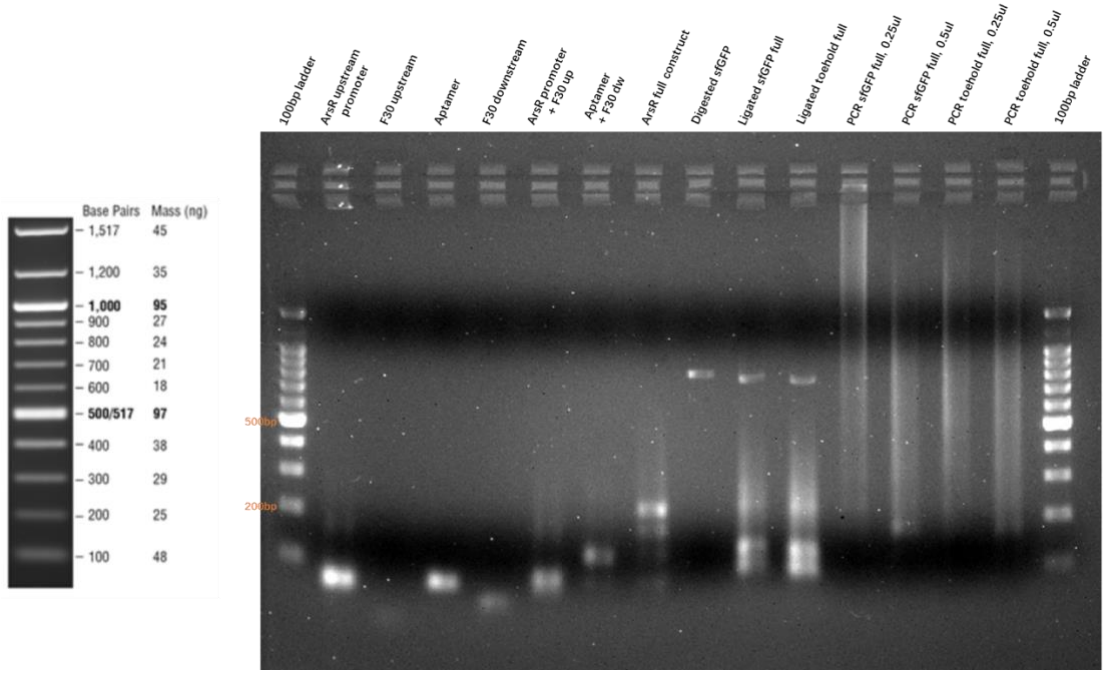
PCR sfGFP full, (0.25ul primers 2ul Sample + 1 Dye + 2 H2O)

PCR sfGFP full, (0.5ul primers 2ul Sample + 1 Dye + 2 H2O)

PCR toehold full, (0.25ul primers 2ul Sample + 1 Dye + 2 H2O)

PCR toehold full, (0.5ul primers 2ul Sample + 1 Dye + 2 H2O)

100bp ladder



New ArsR construct has band at correct size, successful ligation. However, bands for PCR products are still a smear. Ligated toehold switch has similar size with digested sfGFP? Maybe the ligation did work, change the condition for ligation next time.

23/10/2020

Adjusted ligation

	Stock concentration	Final concentration	Volume (ul)
T7 + Toehold	4 uM	55ng	0.159
Digested sfGFP	528 ng	105ng	10ul
T4 ligase buffer	10x	1x	2ul
T4 ligase	400000 U/ml	20 U/ul	1ul
Nuclease free H20			Fill up to 20

Incubate at room temperature for 60 mins and inactivate at 65℃ for 10 mins.

2% agarose with 50ml TBE + 1g agarose + 0.5ul CYBE safe

100bp ladder: 2.5 µl

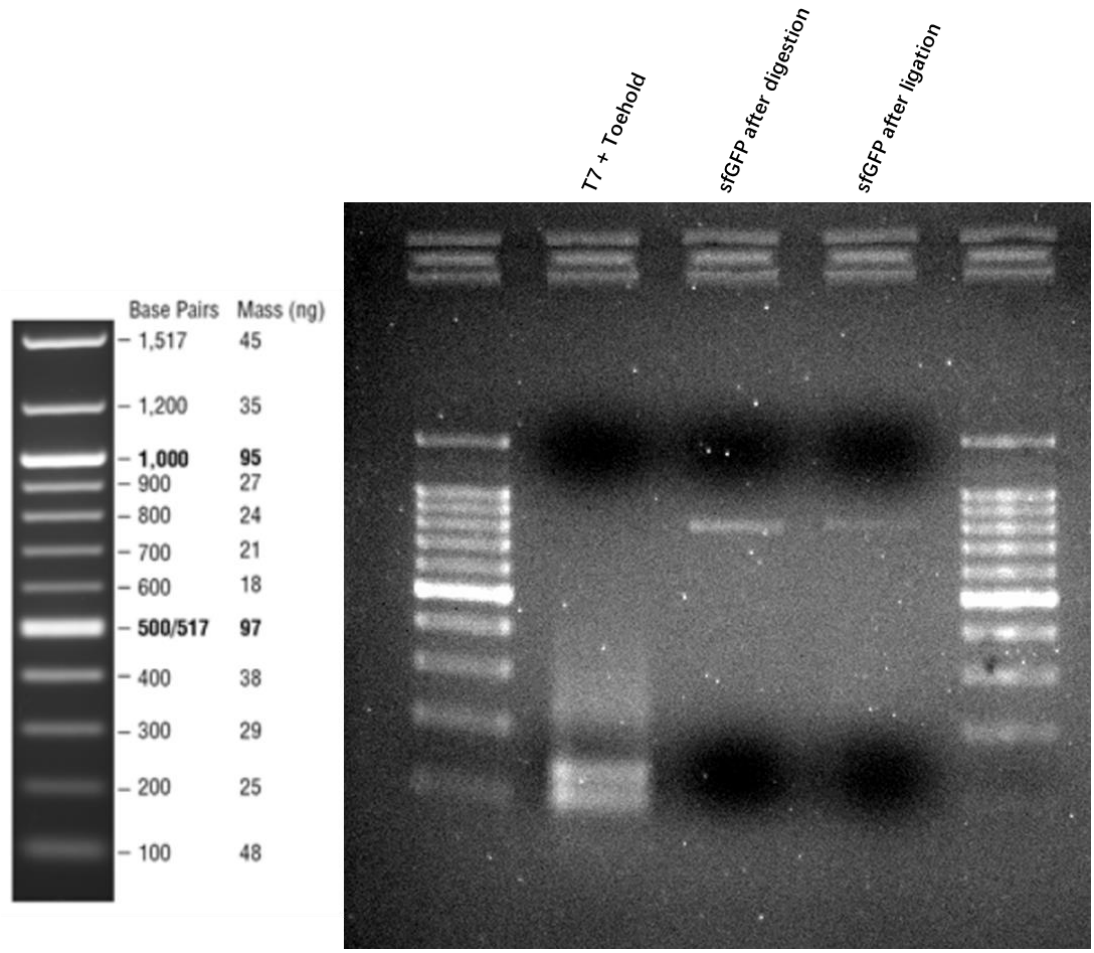
T7+Toehold: 8µM, 100ng, 0.14 µl sample + 1 Dye + 3.86 H2O

Digested sfGFP: 2 µl sample + 1 Dye + 2 H2O

Ligated Toehold: 2 µl sample + 1 Dye + 2 H2O

100bp ladder: 2.5 µl

90V for 35 mins



Ligation still does not seem to be working…

-Riboswitch IVT

Sample	Stock Conc	Final Conc	Blank (ul)	Pos ctl (T7 construct)	Ribo + 7 uM As	Ribo only	Mix
OTRDB buffer	10X	1X	1.5	1.5	1.5	1.5	19.5

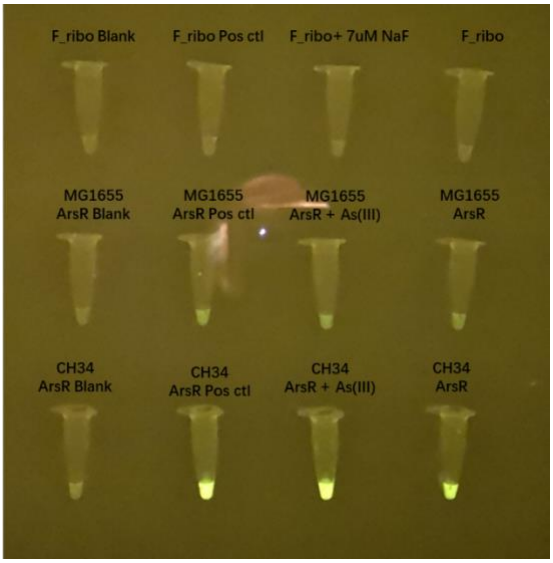
DFHBI fluorophore	200 uM	10 uM	0.75	0.75	0.75	0.75	9.75
NTP	20 mM	2 mM	1.5	1.5	1.5	1.5	19.5
Template	F Ribo: 4uM	100 ng		T7 (4uM): 0.212	0.149	0.149	
NaF	100 uM	7 uM	1.05	1.05	1.05		
MG1655 extract		30% (v/v)	4.5	4.5	4.5	4.5	
Nuclease-free water		Fill up to **15** ul	5.7	5.488	5.551	6.601	

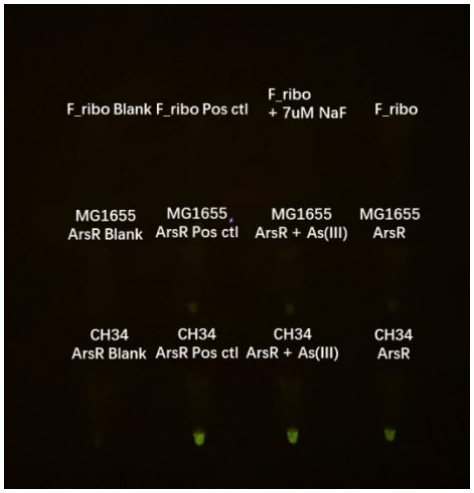
ArsR_T7

Sample	**Stock conc**	**Final conc**	**Blank (ul)**	**Pos ctl (T7 construct)**	Ribo + 7 uM As	Ribo only	Total:
OTRDB buffer	10X	1X	1.5	1.5	1.5	1.5	19.5
DFHBI fluorophore	200 uM	10 uM	0.75	0.75	0.75	0.75	9.75
NTP	20 mM	2 mM	1.5	1.5	1.5	1.5	19.5

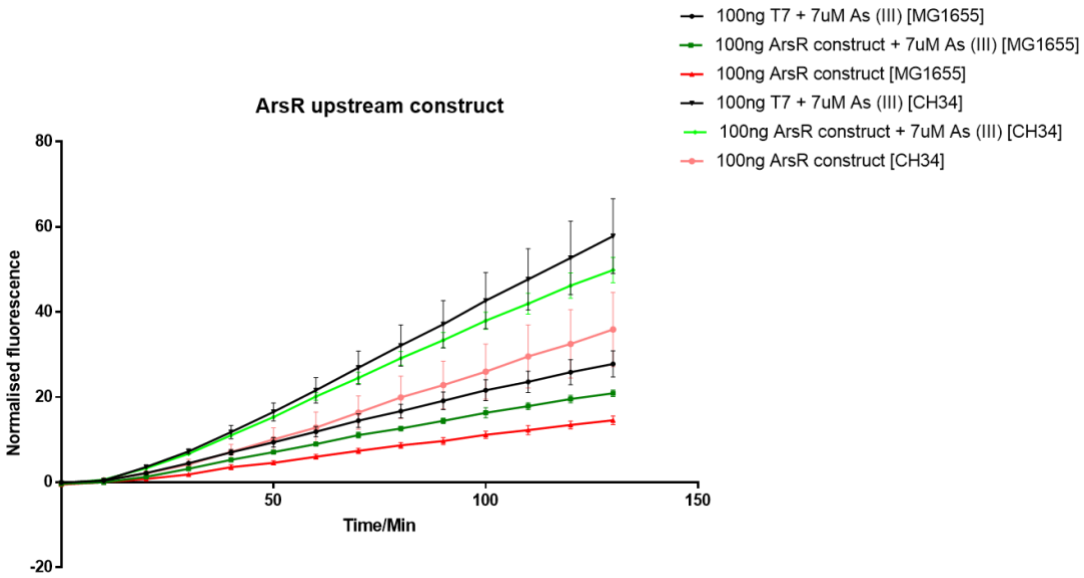
MG1655/ CH34 extract	100%	30% (v/v)	4.5	4.5	4.5	4.5	58.5
Template	TF: 4 uM	100 ng		T7 (4uM): 0.212	0.18	0.18	
As (III)	100 uM	7 uM	1.05	1.05	1.05		
T7 Rpol	50000 U/ml	1.5 U/ul	0.75	0.75	0.75	0.75	
Nuclease-free water		Fill up to **15** ul	4.95	4.738	4.77	5.82	

Results:

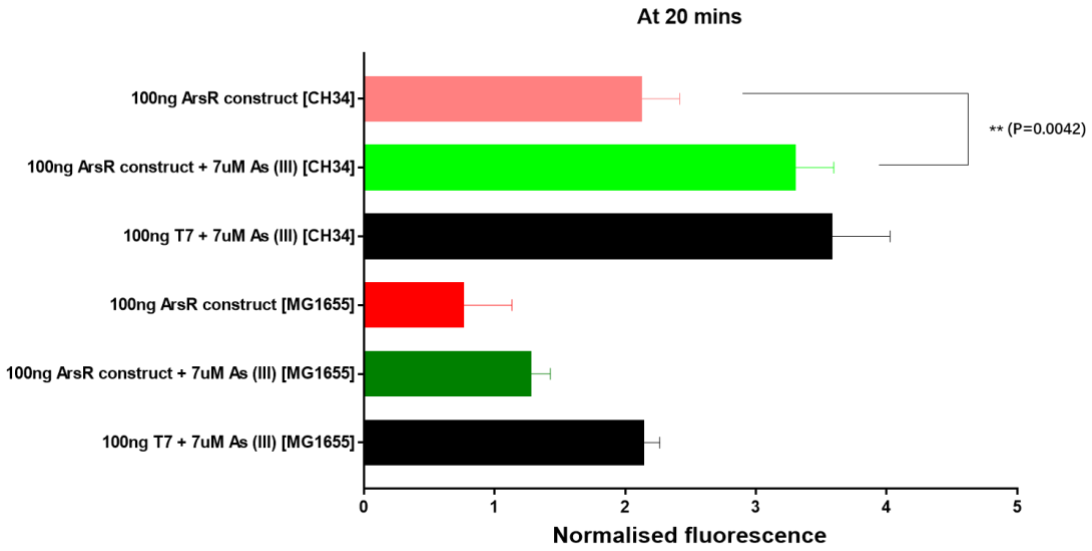




No fluorescence for the riboswitch positive control is because T7 RNA polymerase was forgotten to add

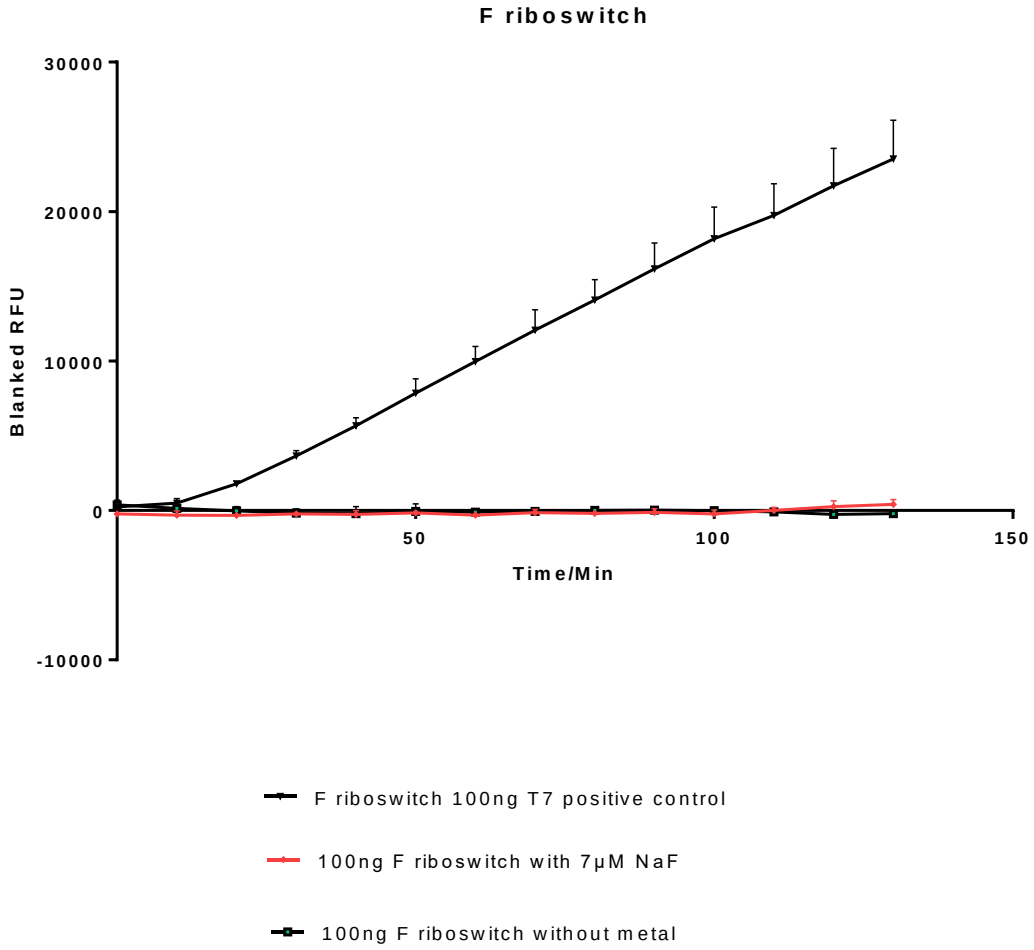


This image illustrates normalized fluorescence readings for the construct with ArsR binding site upstream of the T7 promoter in two cell extracts. In agreement with the previous results, the *Cupriavidus metallidurans* CH34 cell extract has better performance than *E. coli* K12 MG1655 in sensing As (III). The overall fluorescent signal has increased to a level that is almost identical to the positive control.



The significant fluorescent difference in the CH34 extract can be observed at 20 mins in this case.

This construct showed higher transcription leakiness indicated from high fluorescent signal when no As (III) was present. Further optimization, such as reducing the amount of DNA template might be required to solve this problem.



No fluorescence for the F riboswitch. After reading around, this could be due to low metal concentration in the system. Will try higher metal concentration later.