



"dL5* Protein" plasmid prep

Date 29 Nov 2021

29/Nov. **FAP PCR** (~ 800 bp, $0.5 \frac{\text{min}}{\text{kb}}$, $0.8 \mu\text{M} \times 0.5 = 0.4 \text{ min} = 24 \text{ s}$ (time for elongation (72°C)))

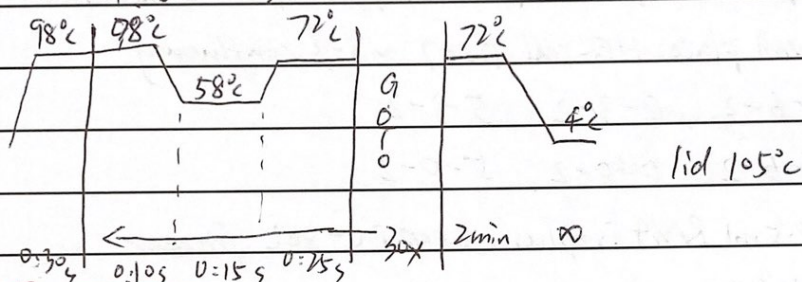
FAP plasmid used: 73205 dL5*-MLS (Template) ~~50 ng~~ (20-60 ng)

Protein PCR primer: FAP Xhd reverse / FAP EcoRI forward (20 mM, 1 μl)
2X Master Mix (12.5 μl)

total 25 μl

(set two PCR tube trials)

Method: JHPHX36



FAP **DNA extraction**

Agarose gel (red) 120V, 20 min: cut fragment ~ 800 bp

Extract DNA with QIAquick Gel Extraction Kit (160051285 Lot)

(170 μg Gel + 510 μl QG $\rightarrow 50^\circ\text{C}$ dissolve \rightarrow + 170 μl IPA \rightarrow column

\rightarrow 500 μl QG \rightarrow 750 μl PE \rightarrow 500 μl PE \rightarrow 20 μl elute (MFH20, 50°C)

FAP **A tailing**

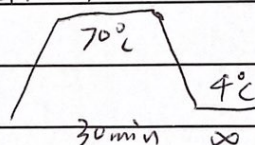
(*can be 0.5 μl)

7 μl eluted DNA + 1 μl Taq DNA polymerase + 1 μl 10x ThermoPol Reaction

Buffer + 1 μl dATP

(also set 2 trials)

Method: ADP-A



FAP **Ligation**

DNA with A tail 4.5 μl + 7 μl 5x^{T4} Ligation Buffer + 0.5 μl Ligation^{T4}
enzyme + 1 μl "T vector" + 2 μl MFH20^{T4} 25 $^\circ\text{C}$, 0 rpm, 1h.

a person to the HK Immigration Department at Mainland
igration Tower, 7 Gloucester Road, Wanchai, Hong Kong
the HK Immigration requires in a couple of week
Once collected the extended
Dep

Date 29 Nov 2021

2

24.5°C
☀️ ☁️ ☔️

29/Nov. Transform

FAP Competent cell: DH5α

LB-agarose plate (with Amp)

37°C 0rpm overnight (22:40 - next day 16:00)

Hoe-DBF
Hoe-PE43-DBF labeling

Hoe-DBF 10/15/μM Hoe-PE43-DBF - 6/3/μM PA - 2 min Green light

6-well plate HEK cell P2+7 ~80% confluency

10-6-2 0-3-2 5-3-2

5-6-2 0-0-2 5-0-2

in 0.5 ml RNA isoplus, keep in -80°C freezer

24.5°C

30/Nov Dot blot and agarose gel.

MG-NLS Dot blot 500ng RNA CuAAC 19/10

1000nM 500nM 250nM 0nM I₂ BrI Ome DMSO

MGOMP

500nM

Agarose Gel 500ng RNA 18/10, 19/10

Before → after CuAAC

Before → after CuAAC

120V, 30min (OK)

I₂ MG-ER

RNA isolation

1000nM/500nM/0nM MG I₂ Ome - 6mm/3mm PA - 3 min light (18/11)

in 40 μl MFHVO

1000-6-3 500-6-3 0-6-3 1000-3-3 500-3-3 0-3-3

882.04 955.68 935.04 944.44 945.92 1078.8

Set long in 75.8ul, 20ng in 40 μl MFHVO aside

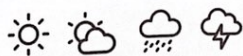
CuAAC, 24°C, 500rpm, 10 min

IPA precipitation column purification

(2nd use)

1000-6-3 500-6-3 0-6-3 1000-3-3 500-3-3 0-3-3

495.76 473.84 467.64 452.20 460.20 432.20

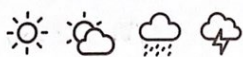


Date 30 / Nov / 2021

③

30/Nov	<u>Dot blot</u>	1000-6-3	500-6-3	0-6-3				
21/11-ER	500ng	1000-3-3	500-3-3	0-3-3				
	(membrane dry 1hr before loading RNA, not good)							
	<u>Agarose Gel</u>	1000-6-3	500-6-3	0-6-3	1000-3-3	500-3-3	0-3-3	Marker
	500ng							
	120V, 30 min							
FAP	Pick 7 <u>monoclonal</u> from trail 1 (agarose LB plate A+)							
	Pick 7 monoclonal from trail 2 (agarose LB plate A+)							
	In 3ml A+ LB buffer, 37°C, 200rpm overnight (20:00-10:00)							
1/Dec.	<u>DNA Extraction</u> of 14 monoclonal.							
23°C	1ug DNA + 0.5ul EcoRI + 0.5ul XhoI fragment, run agarose gel (120V, 30min)							
FAP	pick four of them show highest signal for sequencing							
	1-3, 1-4, 2-4, 2-7 (1-5)							
	ng/ul 696 722, 860 890 (20ul for sequencing)							
	642.9 696.45 747.95 658.40 <u>all in right sequence</u>							
2/Dec.	<u>Dot blot</u>							
22.5°C	700ng RNA CNAAC 19/10	1000nm	500nm	250nm	0nm	M40Me		
14-115								
3/Dec.	<u>Cell passage</u>							
22.8°C	ER-5 monoclonal P2 (frozen ^{stock} 20.10.29) in 6-cm plate							
	<u>Plasmid Fragment</u>							
FAP	200ng dL5* (2-7) in 16ul NFH ₂ O							
	1000ng pET28a+ in 16ul NFH ₂ O							
	+ 1ul XhoI + 1ul EcoI + 2ul 10X digest green buffer.							
	37°C, 0rpm, 2h.							
(used)								

④



Date / /

FAP DNA extraction

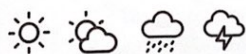
140V, 20 min 1% Agarose Gel

eluted with 20 μ l NF H₂O (50°C)Ligation2 μ l PET28a⁺(cut) + 4 μ l dL5⁺(cut) + 1 μ l T4 enzyme + 4 μ l T4 ligation buffer (5X) + 9 μ l NF H₂O, 25°C, 2h~~need to transform to Rosetta.~~transform to DH5 α 21:00 - next day 12:00, store in 4°C fridge
(low efficiency, only ten monoclonals)4/Dec. ^{Get} Monoclonal ~~2-7~~ from 2-7 (to agarose L-B plate) 18:30 - next day 12:00, store in 4°C fridgeFAP Ligation (as yesterday)5/Dec. Transform to Rosetta cell.22°C Seed ten monoclonals to 3 ml X10 L-B media with K⁺ C⁺, keep
FAP So keep the L-B plate to 37°C again (13:00 - should be K only and hope that still some monoclonals remain)① 19:00 pick 3 monoclonal (from ten) \leftarrow to 16 ml L-B (K⁺) media and 2 to 4 ml L-B (K⁺) media (PET28a⁺ dL5⁺)② pick 1 monoclonal (Tvector + dL5⁺, 2-7) to 16 ml L-B (A⁺) media

19:00 - next day 9:00, 37°C, 240 rpm.

6/Dec. Plasmid isolation (endo-free kit)FAP 3 tubes (16 ml) of PET28a⁺ dL5⁺1 tube (16 ml) of Tvector dL5⁺

(without CS column purification)



Date 6 / Dec / 2021 ⑤

Elute plasmid with 100 μ l NF H ₂ O				
PET28at dL5*	①	②	③	Tvector dL5* (④)
ng/ μ l	82.85	88.75	185.55	558.50

FAP confirm by fragment

500 ng plasmid + 0.5 μ l XhoI + 0.5 μ l EcoRI + 28 μ l 10x digest green buffer
 total 20 μ l, 37°C, 1.8 h, 0 rpm.

agarose gel 140V, 20 min: ① ①-cut ②-cut ③-cut 1Kb DNA ladder ④ ④-cut
 result: ①, ②, ③ cannot be cutted to two fragments. ④ can be cutted to two fragments

hoe-DBF labeling

10 mM hoe-DBF (EG3) (1% DMSO) 800 μ l, 30 min	10	0	10
PA 3 mM or 0 mM, Green light 2-3 min	10	0	0-3

RNA isolation (RNA isoplus), resuspend RNA in 60 μ l NF H₂O, ng/ μ l

10-3-3 (1)	10-0-3 (2)	10-0-3 (3)	0-3-3 (1)	0-0-3 (2)	0-0-3 (3)
903.12	809.14	915.48	897.80	885.52	942.54

DNase + PK: 40 μ g RNA in 80 μ l NF H₂O, DNase 15 min, 37°C, 300 rpm.

37°C, 220 rpm
 (12.70 -

PK 15 min, 42°C, 300 rpm, column purification, elute with 80 μ l NF H₂O ng/ μ l

volume	①	②	③	①	②	③
	75 μ l	77 μ l	63 μ l	80 μ l	80 μ l	78 μ l
conc.	595.45	545.75	498.20	563.05	550.60	573.45

store in -80°C: 3X (5 μ g in 20 μ l NF H₂O)

EG-③ and DMSO-③
 ② or 1X (1.5 μ g in 15 μ l NF H₂O)

EG-③ and DMSO-③
 0X or 1X (10 μ g in 25.83 μ l NF H₂O)

Agarose gel (1%) 120V, 30 min 1.500 ng RNA before DNase + PK

(Two) DBF DMSO

left to right: ① ② ③ ① ② ③ (14)

2) 500 ng RNA after DNase + PK

first test 2) and not very clear two lines

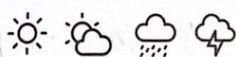
FAP

Fragment of Tvector dL5* (④) 2000 ng plasmid + 1 μ l XhoI

+ 1 μ l EcoRI + 2 μ l 10x Digest green buffer (total 20 μ l), 37°C, 0 rpm, 1.5 h

RNA Extraction: 120V, 20 min, 1% Agarose Gel, cut ~80 bp fragment eluted with 20 μ l NF H₂O

6



Date / /

FAP Ligation

PET28a+ (3/Dec. cutted) 2ul + dL5* (6/Dec. cutted) 4ul + 1ul T4
 enzyme + 4ul 5X T4 ligation Buffer + 9ul H₂O, 25°C, 1000 rpm, 1.5h
 Transform to DH5α in LB plate (K+)

7/Dec. CuAAC of 10ug RNA (hoe-E3-DBF 10nm/0nm, PA 3nm, light 3min)
 at 25°C, 500 rpm, 10 min. Column purification, elute with 20ul N₂H₄

hoe-DBF 10-3-3: 310.24 ng/ul ^{2nd use} 0-3-3: 312.96 ng/ul

Dot blot: 500ng RNA (OK)

ER-5 monoclonal HEK 293T cell labeling imaging

MG

Iz 250nm	OME 250nm	Iz 500nm	Bv1 250nm	OME 1000nm	Bv1 500nm
Iz 1000nm	OME 500nm	Iz 2000nm	Bv1 1000nm	DMSO	Bv1 2000nm

Incubate 20 min, 3min PA, 300 w/m² 3min (660nm)

click reaction with TAMRA-azide (545-azide) at 37°C for 1h.

Result: very low label for ALDOME. Iz is

Similar to probes in different conc. (OME and Bv1)
 slight increased signal in higher conc. (Iz)

FAP

ligated PET28a+ - dL5* in LB plate (K+)

pick 12 monoclonals in 3ml LB media, 37°C, 220 rpm, 19:45 -

100% confluency
 Cell passage: HEK 293T (from JH) 19:5 one 6-cm dish 1:20
 each well 14ml media | two 6-well plates 1:8, 1:16

8/Dec. Plasmid extraction

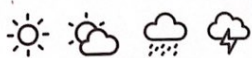
FAP

(home-made P1, P2, P3 buffer)

12 monoclonals yesterday and 2 from 5/Dec. (total 14)
 Resuspend in 50ul N₂H₄, 1ul RNase A, 37°C, 15 min

conc. from 828.4 to 1078.7.

7.



Date 8 Dec. 1 2021

⑦

Fragment (1ul plasmid + 0.5ul XhoI + 0.5 ul EcoRI + 2ul 10x buffer + 16 ul MF H₂O, 37°C, 1 h)

result: all are empty PET 28at vector, it might because of usage of wrong fragment buffer (need use smart instead of digest green)

FAP Ligation

2021. 5. 10 10 fragment PET 28at 0 + 6/Dec. dL5*

2 ul

68 ul

+ 1 ul T4 ligase + 4 ul 5x T4 ligation buffer + 7ul H₂O, 25°C, 2h, 2.5

not enough, so add plasmid and buffer to ligation tube

FAP Transform

DH5α (failed) ~~no~~

Mitosis (HEK 293T from JH) arrest

hoe-DBF 1:8 (yesterday seeded) ~ 50%, 1:16 ~ 20%

17:20 add 14 or 7 ~~ul~~ 200 mM Thymidine (in H₂O) to well (1:8 and 1:16)

19:20 as above (The Thymidine ~~was~~ in UV before adding)

21:20 as above

~~can~~

cell passage: ER-5 monoclonal B 1:20 in 6-cm dish

1:6 in 6-well plates (two) PDL2x 1.5h.

9/Dec. 10-3-3(1), 10-0-3(2), 0-3-3(1), 0-0-3(2) input (1.5ug in 15ul)

23°C

RT-qPCR

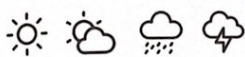
hoe-DBF Qubit4 (10 ~~ul~~ S1, 12ul S2) ~~broad~~

S₁ S₂ 10-3-3(1) 10-0-3(2) 0-3-3(1) 0-3-2(2)

62.57 692.08 322 280 340 352 (broad) ng/ul

62.19 694.21 30.4 26.2 31.8 33.2 (HS) ng/ul

1000 ng (broad) for RT, two trails (in significant C_q change)



Date 13 / Dec / 2021

13/Dec. Cell mitosis (9:30 am) Imaging

7:24 L almost all cell in round for Thymidine (100 nM) + Nocodazole (100 ng/ml)

hoe-DBF Some cells are still in stretched shape and in similar ratio for Thymidine (100 nM) + Nocodazole (50 ng/ml) and only Nocodazole (100 ng/ml)

1. incubate cells with 150 ul 10 nM hoe-E63-DBF for two wells (Thymidine 100 nM + 100/50 ng/ml Nocodazole), other 4 wells with 1 ml HBSS for 30 min

2. Wash with 1 ml HBSS. Cells incubated with hoe-E63-DBF are more fragile than without hoe-E63-DBF (slightly), Most cells floating. Cells without Thymidine treatment looks better.

3. Media 1 ml, 20 min, twice (almost no cell remain for hoe-DBF treated coverslips)

4. Fix for 12 min (4% FPA)

5. 1/2000 hoechst 3 min (not for hoe-DBF labeled coverslips)

6. wash one with HBSS, then mount.

FAP Plasmid extraction (JH made P1 P2 P3)

1 ul RNase A digest for 15 min at 37°C, orpm.

then 2 ul Plasmid + 0.5 ul xhoI + 0.5 ul EcoRI + 2 ul 10x digest Green + 15 ul NF H₂O, 37°C, 0rpm, 1.5 h.

Agarose gel 120V, 30 min: all ~~sample~~ should be right.

20:00 ~~pick~~ pick tube 5 e.coli to a new L-B plate (+) to get monoclonal

ER-MG Cell imaging

By 1 from 250 nM, 500 nM, 1000 nM, 1500 nM, 2000 nM, 0 nM

in 1 ml DMEM, incubate 20 min, then fix 15 min, hoechst 2 min.

wash one with HBSS, mount. imaging right after finishing sample preparation.

Result: similar signal strength

HEK 293T P1
Cell passage (JH): 1:10 and 1:20 in 1.5 ml confocal dish (PDL)



Date 15 / Dec / 2021

13

Cell passage 13:30 { 1:20 6-cm dish
ER-5 monoclonal P6 { 1:20 6-well plate (PDL)
1:4 6-well plate (PDL)
1:10 confocal dish (1ml) (PDL, 2ug/ml puromycin)

FAP Transform to Rosetta (17:30)

1ul dL5⁺-PET28a+ (70.8ng), 37°C, 220 rpm, LB 1ml, 2h

Then seeded to L-B_{plate} (K+C) 20:30 - 9:00 (too many monoclonals)

Cell mitosis

hoe-DBF 18:00 wash confocal dishes with HBSS x 3 times
(40% for 1:20, ~95% for 1:10)

21:00 add 0.5ul 200ug/ml Noc to 1ml media (100ng/ml) to confocal dish
add 10ul 100mM Thy to 6-well plate (~1mM)

16/Dec, 24.5°C Live cell imaging (cell mitosis)

Remove media, replace with 1ml 2.5mM hoe-DBF, 20min.

hoe-DBF Wash once with 1ml media, then incubate with 1ml media for 40min.
Add HBSS with (1/5000 hoe-DBF + 3 or 0mM PA) incubate 3 min before first
imaging. ^{1:20 dish}
comparing to background noise _{1:10 dish}

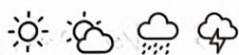
Result: weak DBF signal, no different for dish with/without 3mM PA
(DBF channel: 1250, 1, hoeshet channel: 1250, 2)

Protein expression compare

FAP Pick 12 monoclonal to 4ml LB media (1:1000 K + 1:4000 C), 37°C, 220rpm
except tube 5, which is much lower
12:20 - ~20:20 : all 12 in similar OD (by neck eye), test tube 3:
OD = 1.10 by adjust time and tube position "1" or "2"

20:45 add IPTG (0.1ul to 1ml, 9.1ul to 3.8ml) to remain solution.
21:00 to 16°C, 150 rpm. - next day 15:00 (18h)

12



Date / /

24°C 14/Dec. (RT-qPCR) 344 328
 hoe-DBF (Qubit4 tested 9/Dec. 10-0-3 and 0-0-3)
 1000 ng for RT, 2 trials

ER-5 (Cell imaging dual-labeling)

EM-MG + Hoe-E43-DBF 150ul 5mM 30 min

hoe-DBF Media 20 min, MgI₂OME 500, 1000 or 2000 uM 20 min, PA 3mM, Green 1 min

500	1000	2000	red 1 min (Green 60s)
500	1000	2000	Red 3 min (Green 65s)

Result:

Cell passage 17:00

HEK293T P11 (from JH) 1:20 6-cm dish

1:80 6-well plate (PDL) "no coverslips"

(Plasmid amplification)

FAP Pick 2 monoclonal pET28at-dL5⁺ from LB plate (+) to 16 ml media.
 37°C, 220 rpm, 17:20 -

Cell mitosis (21:00)

1:10 and 1:20 seeded 24h before ago (in 1.5 ml) in confocal dishes.

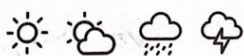
15/Dec. (Plasmid extraction from DH5α)

24°C "质粒小提中量试剂盒" ① ②

FAP elute with 100 ul MFH₂O 70.8 61.8 ng/ul

1000 ng Plasmid + 0.5 ul EcoRI + 0.5 ul XhoI + 2 ul Green digest, 37°C, 1h.

120V, 30 min Agarose Gel: Both are right



FAP

Fragment

pET28a+ (from 201.5) 1000 ng ~~XhoI~~ XhoI 1ul, EcoRI 1ul 10x fast digest 2ul
 dL5* (from 201.12.6) 2000 ng XhoI 1ul EcoRI 1ul 10x fast digest 2ul
 to 20ul total, 37°C, 0 rpm, 1.7h.

Agarose gel 140V, 20min

Plasmid ~~re~~ extraction with Quik kit, elute plasmid with 20ul
 NFIH₂O (50°C)

FAP

Ligation

2ul pET28a+ fragment 1ul ligase
 + 2ul 10x ligation buffer + 1ul NFIH₂O
 4ul dL5* fragment (from pLip L1)
 25°C, 0 rpm, 2h

FAP

Transform to DH5α (18:00), 2:00 to the LB plate (K+)

Cell passage: ER-5 monoclonal R 5:1 to 6-cm dish

1:1 and 1:6 6-well plates (PDL)

Hoe-DAP

Cell mitosis

9:30 pm 100 nM Thymidine to 4 wells. (~20%)

12/Dec

17:00 (~40% - 50%) wash 4 wells 3 times with ABSS, then 1.5 ml media

24°C

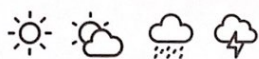
2:00 3 wells with 1.5 media (100 ng/ml Nocodazole)

Hoe-DAP

3 - - - - - (50 ng - - - - -)

FAP

E. coli in LB plate ~~are~~ isn't in monoclonal, just pick 6 ^{merged} ~~merged~~ monoclonals
 to 3 ml media (K+), 37°C, 220 rpm, 2:00 - 9:30 am.



Date / /

22/Feb Protein purification (A15-VI)

17°C

FAP

dL5* - flask 3 and 4 (~450ml, second large scale culture)

1. Thaw from -80°C in R.T. Add 20ml binding buffer (lysis + 5% glycerol) to ~23ml in total. Suspend with metal spoon to milky solution

2. Add 3ul Millipore rLysozyme (30KU/ul), Rotate at R.T. for 15min

3. ~~Freeze~~ Add 1.6ml PMSF (100mM, 1:1000, should be 230ul)

4. Freeze at liquid Nitrogen (4min) / Thaw at 37°C water (4min) x 2 times. Then Add 20ml binding buffer more Freeze / Thaw again

5. Sonicate with small tip. 5s on / 5s off, 20% Amp. 30min

6. 15,000G, 30min. ~~filter with~~ through 0.22um filter (set 50ul aside)

7. Binding with 500ul new Ni Sepharose 6 Fas flow (pre washed with 2ml binding buffer x 3 times) at 4°C, 12h.

8. Elution

0% (1) 0% (2) 0% (3) 4% (1) 4% (2) 4% (3) 6% (1), 6% (2), 6% (3), 8% (1), 8% (2), 8% (3), 10% (1), 10% (2), 10% (3) 15% (1), 20% (1), 30% (1), 50% (1) 5ml, 50% (2), 50% (3), 100% (1) 100% (2), 100% (3), 100% (4) 1.5ml

9. For debris, ~~sonicate~~ add 15ml binding buffer, sonicate 20min. 15,000G, 30min, ~~filter with~~ through 0.22um filter (set 50ul aside)

10. Bind with reused beads (~500ul used twice, ~450ul used once) for 2h.

11. Elution

(0% (1), 0% (2), 0% (3), 4% (1), 6% (1), 8% (1), 10% (1), 10% (2), 15% (1), 20% (1), 20% (2), 30% (1), 50% (1), 50% (2), 100% (1), 100% (2), 100% (3), 100% (4)) 1.5ml



Date / /

15% SDS-Page Gel.

5ul 4x loading buffer with SH

8 Elution: 15ul protein, 2ul Marker ✓ 90°C, 10min

① Marker, filtered(2), flowthrough(1), 0(1), 0(2), 0(3), 4(1), 4(2), 4(3),
6(1), 6(2), 6(3), 8(1), 8(2), 8(3)② 10(1), 10(2), 10(3), 15, 20, 30, 50(1), 50(2), 50(3), 100(1), 100(2),
100(3), 100(4), filtered(2), MarkerWash two column: H₂O 2ml x 2, 6M Glycine 2ml x 2, H₂O 2ml x 2, 20% EtOH
2ml x 2

23/Feb 15% SDS-Page Gel

Store solution

17°C 11 Elution: 15ul protein, 2ul Marker.

FAP ① Marker, filtered(2), flowthrough(2), 0(1), 0(2), 0(3), 40, 6, 8, 10(1)
② 10(2), 10(3), 15, 20(1), 20(2), 30, 50(1), 50(2), 100(1), 100(2)Protein Purification

d15* flask 1, 2 (~200ml, first round large scale culture)

1. Thaw from -20°C in R.T. Add 20ml binding buffer to 23ml total!

Mix by pipetting

2. Add 4ul Millipore lysozyme, Rotate at R.T. for 15min

3. Freeze (4min) / Thaw (~8min) x 3 times

4. Add 230ul 100mM PMSF (1/1000)

5. Sonicate with small tip. 5s on / 5s off, 20% Amp. 30min

6. 15,000 G, 30min, through 0.22um filter

7. Binding with 500ul reused (yesterday) beads. 4°C for 1.5h.

8 Elution:

(0% (1), 0% (2), 0% (3), 4% (1), 4% (2), 4% (3), 6% (1), 6% (2)

7(3) (8% (1), 8% (2), 10% (1), 10% (2), 50% (1), 5ml, (50% (2), 50% (3)

(50% (4) 4ml, (50% (5), 100% (1), 100% (2), 100% (3) 1.5ml)

9. For debris add 15ul binding buffer, 150ul PMSF, sonicate 30min.



Date 15 / Dec. / 2021

Cell passage 13:30 { 1:20 6-cm dish
 ER-5 monoclonal P6 { 1:20 6-well plate (PDL)
 1:4 6-well plate (PDL)
 1:10 confocal dish (1ml) (PDL, 2ug/ml puromycin)

FAP Transform to Rosetta (17:30)

1ul dL5*-PET28a+ (70.8 ng), 37°C, 220 rpm, LB 1ml, 2h.

Then seeded to 6-plate (K+C) 20:30 - 9:00 (too many monoclonals)

Cell mitosis

hoe-DBF 18:00 wash confocal dishes with HBSS x 3 times
 (40% for 1:20, ~95% for 1:10)

21:00 add 0.5ul 200ug/ml Noc to 1ml media (100 ng/ml) to confocal dish
 add 10 ul 100 mM Thy to 6-well plate (~1 mM)

16/Dec. live cell imaging cell mitosis

24.5°C

Remove media, replace with 1ml 2.5 nM hoe-DBF, 20 min.

hoe-DBF Wash one with 1ml media, then incubate with 1ml media for 40 min.

Add HBSS with (1/2000 hoe-DBF + ^{1:20 dish} 3 or 0 mM PA) incubate 3 min before first
^{1:10 dish}
 imaging. comparing to background noise

Result: weak DBF signal, no different for dish with/without 3mM PA

(DBF channel: 1250, 1, hoechst channel: 1250, 2)

Protein expression compare

from Ulrike

FAP

→ 1-43 in 5 ml LB media
 Pick 12 monoclonal to 4ml LB media (1:1000 K + 1:4000 C), 37°C, 220 rpm
 → except tube 5, which is much lower

12:20 - ~20:20 : all 12 in similar OD (by neck eye), test tube 3:

OD = 1.10 by adjust time and tube position "1" or "—" "

20:45 add IPTG (0.1ul to 1ml 0.9.1ul to 3.8ml) to remain solution.
 21:00 to 16°C, 150 rpm. - next day 15:00 (18h)



Date / /

29/Dec. Mitosis:

12ul 100mM Thymidine to each confocal dish (9:10 p.m.)

30/Dec. FAP (dL5*)

Rosetta ~~tra~~ (before inducing) 20ul (tube 4) to 6 ml media (k+c)

FAP

37°C, 220rpm, 10:30 - ~~18:00~~ ^{next day} 11:00. Then to 4°C fridge. ^{Too long. Keep in 4°C fridge.}

For purified dL5*: in 12% SDS Page Gel

①: Marker, lysate, w1, w2, w3, w4, w5, w6, w7, w8, w9, w10, w11, w12, w13

Marker 100ul

②: w14, w15, w16, w16', w17, E1, E2, E3, wash A, w7, Marker, lysate, Flow-through (1)

lysate 2ul

③: Marker, lysate, F-T, w1, w2, w3, w4, w5, w6, w7, w8, w9, w10, w11, w12

others 15ul

④: w13, w14, w15, w16, w17, E1, E2, E3, Marker

Mitosis

18:30 wash 3 times with DPBS, then 1ml media.

9:21:30 0.5ul 200µg/ml Nocodazol to each dish. (100µg/ml)

31/Dec. Mitosis

Hoe-DBF

~ 10:40 10µM hoe-EG3-DBF 30 min. 1ml media 20 min, 1ml media 4 min
then 1/1000 hoechst in colorless DMEM

Result: weak localization, similar to 2.5µM test

cell imaging

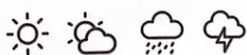
Mn-ER

FAP

Mn-ER 1µM 0.5ul 20 min, 2mM FzA2ide or PA 3 min. Red 1 min

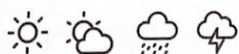
FAP (dL5*)

Rosetta from 30/Dec. 20ul to 6 ml culture (k+c), 9:11:00 - 18:00
Then to 4°C fridge



Date 2 Jan/ 2022

	Cell passage:
	new frozen stock ER-5 P ₂ in 6-cm dish
2/Jan.	B Prepare culture media (auto clave)
FAP	5mg (5mg Powder in 200 ml H ₂ O) X 3 (1, 2, 3) (6g Powder in 300 ml H ₂ O) X 1 (4)
3/Jan	dL5* culture (large scale)
FAP	200ul dL5*-Rosetta (3/dec.) to flask 1, 2 and 3 300ul to flask 4 : 37°C, 220rpm, 9:30-18:00 (Remain dL5*-Rosetta, add 1.2 Volume 40% Glycerol, two tubes to -78°C, box 4) 37°C, 0rpm : 18:00 - next day 9:30 37°C, 120rpm : 9:30-11:40, (10:10 flask 2 OD = 0.526) then gose to 17°C, 0rpm (11:40 flask 2 OD = 0.818) 17:30 - next day 9:30 (16h) 17°C, 150rpm.
5/Jan	remove culture media, collect to pellet to two 50ml tube (each ~ 2g wet weight), keep store in -80°C, lowest lattice cabinet
	Cell Passage:
	ER-5 P ₃ { 1:10 6-cm dish 1:8 6-well plate (PDL, coverslips)
	HEK (JH) two confocal dishes (PDL) 1:8
4/Jan	Cell pellette from flask 3, 4, 400ml into culture.
FAP	Wet pellette ~ 2.5g Purify dL5* with new buffer and new beads.
SDS-PAGE	result: flow through (F-7) and S-f looks similar. and several lines for gradient eluted product, need denature before next time.
	Cell mitosis: 21:00 7ul 100mM Thy to each confocal dish (~50% confluency)



Date / /

^{2nd}

11/Jan. UV-Vis of MG10ME in 10 ml ACN, 1 ml DPBS

	Time/min	au.	Time/min	au.	Time/min	au.
UV-Vis	0	0.698	15.5	0.660	45	0.614
MG10ME	3	0.694	18	0.654	50	0.608
	6	0.686	21	0.651	60	0.597
	9	0.677	25	0.645	90	0.581
	12	0.668	30	0.634	120	0.572
		0.660			180	0.566

dLT⁺ - purification

FAP flow-through ~ 25 ml from -20°C, 2022.1.4

Beads: 450 µl used + 100 µl new, prewash with 2 ml ^{Tris} lysis buffer X3.

Binding: 4°C, 2h.

Elution: W1, W2, W3. 8 ml lysis buffer

W4, W5, W6, W7, W8, W9, W10, W11, W12, W13, W14, W15, W16

W17, W18, W19, W20, W21, W22, W23, W24, W25, W26

50% 100% 0% 94% H₂O

Wash: 1 ml 6M Glycogen X3, 1 ml H₂O X3, 20% EtOH 1 ml

Store in 20% EtOH, 4°C

dLT⁺ - purification

FAP insoluble debris from -20°C, 2022.1.4

Thaw/Freeze Cycle: 5 min dry ice, 2 min 37°C water bath, X 5 times

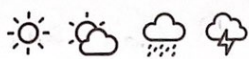
Sonicate: 40% Amplitude, 2s on, 5s off, 3 min, X 5 times

Centrifuge: 15,000 x G, 4°C, 30 min

Binding: ~12 ml, 600 µl Beads (prewash with 2 ml lysis buffer X3), 4°C, 2h

Elution: as above

Cell Passage: HEK 293T 12+2 Gatte 1:20 in 6-cm dish



Date / /

Micro in colorless DMEM, capture images through 35 min:
show similar level through 35 min.

Mitosis

17:00 wash 3 times with DPBS, then replace with 1 ml media
20:50 add 0.5 ml 200 μ g/ml Nocodazole to each dish

13/Jan. ~~9:40~~ Mitosis, live cell imaging

9:40 remove media, wash with DPBS, then add 500 μ l 10 or 2.5 μ M
hoe-EH3-DPF. incubate 30 min. Wash twice with media, 20 min,
Mitosis each. then DPBS with 1/1000 Hoechst 10 min.
Result: both are not very good.

Protein dialysis ^{at 4°C} (flask 3.4 - first round)

FAP

One: in low conc. and not that pure

From main round: W5, W6, W7, W8, W9, W10, W11, W23

1) flow-through: W15, W16, W18, W19

2) insoluble debris: W13, W14, W20, W21, W22

Another: in high conc. and pure

From 1. main round: W12, W13, W14, W15, W16, W17, W18, W19, W20, W21, W22

1) flow-through: W17

2) insoluble debris: W15, W16, W17, W18, W19

start 2:00 pm.

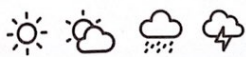
dialysis: DPBS 500 ml 30 min \rightarrow DPBS 500 ml 40 min: high conc. show

some solid \rightarrow DPBS: Elution buffer = 10:1 \rightarrow 10:3 \rightarrow 1:1 =

still some solid, so ~~at~~ transfer the protein in high conc. to another row

membrane with more ^{Elution} buffer to dilute it. Then dialysis in reduced urea

22:30 add 1.5 ml 100 mM TMSF (protein inhibitor). stirring overnight



Date / /

PL (3min), PLE (4min), PL/PLE (1h), PL/PLE (3h) ^(3.1h in dead)

PL of MgI_2 / 5mM NLS-dLS* = titration

800 μ l NLS-dLS* ~~(50min)~~

↓ + 2 μ l 1mM MgI_2 , incubate 3min

2.5 mM MgI_2

↓ + 2 μ l 1mM MgI_2 , incubate 3min

5 mM MgI_2

↓ + as above

7.5 mM MgI_2

↓ as above

10 mM MgI_2

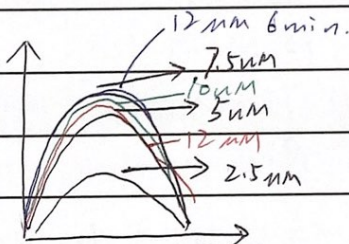
↓ as above

12.5 mM MgI_2

↓ 3 min more

12.5 mM MgI_2 (6min)

Abs of probes in NLS-dLS* (5 μ M) = 3h.



NLS-dLS* Protein Transform

NLS-dLS* (cell = Rossetta (ED3))

93.76 ng/ μ l. 2021.5.21

Plasmid: pET28a+ NLS-dLS* (tube 2)

to Agarose plate (K+C) (18:30 - Next day 13:00, 37°C, 0 rpm)

12/3 Monoclonal selection and Protein Expression

NLS-dLS* pick 8 monoclonals to 8 15ml-tubes ~~containing~~ containing 6ml

21.5°C LB media (K + 1/4000 C), 17:15 - 19:05, 37°C, @220rpm)

19:05 → 20:30 R.T. 0 rpm, 20:30 - 10:30, 100 rpm, 37°C, 10:30 -

next day

(40 μ l to tube 2)

16:20, 220 rpm, 37°C, 16:20 add 60 μ l 100mM IPTG to 6ml solution, 16°C,

14:20 tube 2 : 0.578 OD, 15:20 tube 2 : 0.694 OD.

150 rpm.



Date / /

14/3 9:30 combine all, set 1ml aside, remove ~~set~~ media, keep in -20°C

Protein dialysis

~~DL5~~^{*} 3

DL5^{*} 3 ~ ³⁵~~34~~ ml in -80°C freezer.

13:30 dialysis in 180ml lysis buffer + 60ml elution buffer + 200ml DPBS (home-made, pH 7.4), stirring ⁱⁿ ~~at~~ 4°C fridge

14:35 replace 200ml with fresh DPBS

15:30 replace @ 200ml with fresh DPBS.

16:30 as above

17:30 ~~as above~~ replace all with fresh DPBS

18:40 go through 0.22 μm filter (+12ml DPBS move)

Concentrate: Device Vivaspinn 20, 5,000 MWCO (5KD)

~13 ~~ml~~ 8,000 G 10 min (remove ~3 ml)

~13 ml 8,000 G 15 min (remove ~2.8 ml)

~10 ml 8,000 G 20 min (remove ~2.6 ml)

collect ~26 ml

{ 5ml X 4
1.6 ml X 2 in -80°C box blue
~1.2 ml X 2
100 μl X 1

tube 1.4 still in clear at 19:30

~~14~~¹⁵/3

Monoclonal selection and protein expression

6 monoclonals to 4ml media X 6 tubes (K+ $\frac{1}{4000}$ C)

9:35 - 17:30, 37°C , 220 rpm. (2, 3, 5, 6), 17:30 - 19:00, R.T

19:00 tube 2 OD = 1.233, take 200 ml aside (in 4°C)

1ml tube 2 before inducing

other Add IPTG (2.4 μl to 1ml), 19:30 - ^{next day} 11:30, 16°C , 150 rpm
(14h)



Date / /

BCA Assay: 37°C, 1.5h.

dL5*3 (DPBS)	A	B	C	D	E	F	G	H	I	dL5*3 DPBS	DPBS
mg/ml	2	1.5	1	0.75	0.5	0.25	0.125	0.0625	0	0.3559	--
										0.3837	
										0.3636	
										$\bar{O} = 0.3677 \text{ mg/ml}$	
										$= 11.49 \mu\text{M}$ (32KD)	
										(11.26 μM for 32.65KD)	

Fluorescence (HOC)

MG-H₂, MG-^{HBr}~~Br~~, MG-Cl₂, MG-Br₂, MG-I₂, MG-I₂ ethyl1mM: 86 μl 5mM stock in DMSO + 24 μl ACN \Rightarrow 30 μl

dL5*3 (dialyzed in DPBS)

5 μM : 2.828 ml 11.49 μM stock + 3.671 ml DPBS (pH 7.4) \Rightarrow 6.5 ml

BSA control

5 μM : 1.925 mg in 7 ml DPBS (pH = 7.4) \Rightarrow 7 ml

MG-BSA:

UV-Vis: 10 mm Ok, 3h.

PL, PLE: 1 h. (slit 5/5 nm)

MG-dL5*:

UV-Vis: 10 mm 3h

PL, PLE: 3 min, 45 min, 1h 45 min, 1h 10-30 min. (slit 5/5 nm)

90°C, 10 min denature

15/3 SDS-Page Protein expression (15 μl + 15 μl 2x loading, 2 μl Marker)2 before inducing, 2, 3, 5, 6, ^{13/3 (1-8)} Markerwhen cooling down, ~100 μl into tube)

result: 15% SDS-Page is not suitable

Protein expression (large scale) (300 ml x 2)Each: 75 μl CTC (1/4000), 300 μl K⁺ (1/1000 JH), 50 μl tube 2before inducing. Remain ~80 μl tube 2 before inducing to 1 ml 5% Glycerol.
Store in -80°C (orange box)



Date / /

37°C, 150 rpm, 19:00 - 11:45

16/3 9:30 flask 2: 0.240 OD, 11:45 flask 2: 0.796 OD

12:30 add 720 µl IPTG (100 mg/ml) to 300 ml culture.

12:30 - 16:50, 150 rpm, 16°C.

Kd

16:50 - 9:30 ^{next day}, 150 rpm, 16°C, 16.5 h.

remove media, ~1.6 g for each flask, save in -80°C

Binding affinity (MG-H₂)0.2 nM → 1000 nM (3 trials) MG-H₂, 50 nM NLS-dLS*

Kd = 15.6 nM, n = 1.26

17/3

MGHBV

0.2 nM → 2000 nM MG-I₂ and MG-Cl₂ ✓ (yesterday prepared stock)

Kd

50 nM NLS-dLS*

Kd ≈ 200 - 300, not fitting very well (MG-I₂ and MG-Cl₂)

MGHBV Kd ≈ 90 nM

0.2 nM → 2000 nM MG-Br₂, MG-I₂ ethyl, 50 nM NLS-dLS*

Kd ≈ 80 nM

21/3

0.2 nM → 2000 nM (add 300 nM sample) MG-I₂, MG-Cl₂.

50 nM NLS-dLS*

Kd

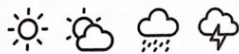
Kd ≈ 200 - 300, MG-Cl₂ not fit very well.Prepare new 0.2 nM → 2000 nM stocks: MG-I₂, MG-Cl₂

50 nM NLS-dLS*

Kd ≈ 100 - 150 nM, fit well

7/3 lyophilized NLS-dLS* (1 ml), store in R.T. for 2 weeks.

Resuspend in 1 ml dH₂O to (assume) 23.87 µM (cannot dissolve well)



Date / /

S₁ S₂ dsDNA (HS Kit ~~dsDNA~~)

56.82. 32488 16.2 ng/ul

H₂O

~~200 ng~~ 100 ng dsDNA (6.17 + 3.83)

1 kb (1/10 dilute) (3 + 87)

20 kb (1/10 dilute) (2 + 8)

1 kb (1/100 dilute) (1 + 89)

100 V, 30 min + 20 min : signal of dsDNA is much higher than Mon
dsDNA ~ 200 - 300 bp

24/3 Protein (NLS-dLS*) Purification

22°C 1. Combine 300 + 300 ml culture collected e.coli: pellet.

2. Suspend in 50 ml buffer A (tris) with 1 min pmsf

3. Sonicate 15 min (5s/5s, 20% Ampt)

NLS-dLS* 4. 10500 G, 30 min, 0.22 μm filter.

5. Bind with 800 ul New Ni fast beads. for 1h.

6. Elution.

W1	W2	W3	W4	W5	W6	W7	W8	W9	W10
0%	0%	0%	1%	2%	3%	4%	34%	48%	5%
5ml	5ml	5ml	5ml	5ml	5ml	1.6			

W11	W12	W13	W14	W15	W16	W17	W18	W19	W20
5%	6%	68%	8%	10%	10%	10%	20%	20%	50%

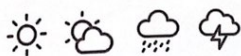
W21	W22	W23	W24	W25
50%	50%			

7. The flow through from supernatant, binding with twice used beads ~ 1.2 ml for 2h.

8. Elution.

W1	W2	W3	W4	W5	W6	W7	W8	W9	W10
0%	0%	1%	2%	3%	4%	4%	5%	58%	68%
6ml	6ml	6ml	6ml	6ml	1.6ml	-	-		

W11	W12	W13	W14	W15	W16
8%	10%	20%	20%	50%	50%



Date / /

9. For debris, sonicate 30 min, centrifuge 30 min (~ 35 ml), bind with 500 ml new beads for 1 h.

10. Elution

W1	W2	W3	W4	W5	W6	W7	W8	
0%	0%	1%	2%	2%	3%	3%	4%	
6ml	-	-	1.6	-	-	-	-	
W9	W10	W11	W12	W13	W14	W15	W16	W17
4%	5%	6%	8%	10%	20%	20%	50%	50%

11. The ~~debris~~-flow through from debris, bind with 800 ml reused beads for 1 h.

12. Elution

W1	W2	W3	W4	W5	W6	W7	W8
1%	2%	3%	3%	4%	5%	5%	6%
5 ml	5 ml	1.6 ml	-	-	-	-	-
W9	W10	W11	W12	W13	W14	W15	
8%	10%	10%	10%	20%	50%	50%	

13. The Flowthrough of flowthrough from supernatant, bind with the three times used beads for 1.6 h.

14. Elution

W1	W2	W3	W4	W5	W6	W7
1%	2%	3%	4%	50%	50%	50%
6 ml	4 ml	4 ml	1.6 ml	-	-	-

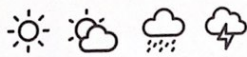
Save all in -20°C .

Take 10 μl out for SDS-Page (8%)

From black - purple - red - green - purple - P, S, FTcs, FT-FTcs, FT-FT-FTcs, debris, FT-FT(debris)

First 4 gels: 80V 40 min, 120V, 60 min

Last 2 Gels: 80V 20 min, 110V, 60 min + 40 min



Date / /

1% Agarose Gel

10-0-3 (2) 71.2 ng/ul, 200 ng (2.8 ul + 2.2 ul NF H₂O)

1 Kb DNA Marker (0.5 ul + 4.5 ul NF H₂O)

120 V, 300 min : Good Quality

26/3 Protein Dialysis

Combine (pure):

W13 W14 W15 W16 W17 W18 W19 W20 W21

0.6% 0.8% 1.6% 1.0% 1.2% 2.0% 2.0% 5.0% 5.0%

W12 W13 W14 W15 W16

1.0% 2.0% 2.0% 5.0% 5.0%

W13 W14 W15 W16

1.0% 2.0% 2.0% 5.0%

$23 \times 1.6 = 36.8$

W11 W12 W13 W14 W15

1.0% 1.0% 2.0% 5.0% 5.0%

Combine (not that pure)

W10 W11 W12 W11 W12 W10 W5 W6 W7

5% 5% 6% 8% 8% 10% 5% 5% 5%

~~W10~~ $9 \times 1.6 = 14.4$

purple sample
very high conc.

100 ml buffer B + ⁵⁰⁰~~450~~ ml DPBS, 11:45 - 13:25

replace 300 ml buffer with 300 ml DPBS 13:30 - 14:30

replace all with 600 ml DPBS, 14:30 - 15:45

replace half with DPBS, 15:45 - 18:00

replace all with DPBS, 18:00 - 20:00

$24 + 10 + 9 + 1 = 44$

NLS-dLS*: 12 ml x 1, 6 ml x 4, 5 ml x 2, 1.6 ml x 45, 1 ml x 1

NLS-dLS* not pure: 12 ml x 1



Date / /

28/3	<u>BCA Assay</u>		NLS-dL5* diluted	NLS-dL5* (1/2 dilute)	NLS-dL5*	not pure NLS-dL5* diluted	not pure NLS-dL5*
	A B C D E F G H I		0.4089	0.5583	0.9983	0.1521	0.1913 mg/ml
	2 1.5 1.0 0.75 0.5	55KD	7.43 μ M	10.15 μ M	18.15 μ M	2.76 μ M	14.39 μ M

Concentrate of NLS-dL5*

NLS-dL5*

6 ml \times 4 = 24 ml, rinse with 1 ml DPBS,

Vivaspin 20, 10 kDa MWCO, ~~600~~ (pre-rinsed with Δ H₂O)

1. 12 ml NLS-dL5* to concentrator, 5000 G, 5 min: remove ~2-3 ml

2. 12 ml NLS-dL5* to concentrator, 5000 G, 10 min: remove ~4 ml

set 6 ml aside (to 15 ml tube, labeled as (2))

3. Combine remain FAP (~11 ml), 5000 G, 6 min; remove ~2.2 ml

6 min more: remove ~2 ml

set 6 ml aside (to 15 ml tube, labeled as (1))

remain ~0.9 ml, add ~220 μ l DPBS more, labeled as NLS-dL5* high conc.

4. rinse the concentrator with Δ H₂O, then 70% EtOH.

BCA Assay

concentrated

A B C D E F G	NLS-dL5*	NLS-dL5* (1) 6 ml	NLS-dL5* (2) 6 ml	NLS-dL5* high conc. ~1.2 ml
	1.0729	2.1412	1.5520	1.9501 mg/ml
	19.51 μ M	38.93 μ M	28.22 μ M	35.45 μ M

Fluorescence of new NLS-dL5*

For FA

assume 18.15 μ M, 5 μ M FAP NLS-dL5* + 5 μ M M412 ~ 600 a.u. 5/14 5/5

NLS-dL5*

5 μ M NLS-dL5* + 5 μ M 2h aged M412 ~ 200 a.u. + 5 μ M

M412

M412 more, to ~600 a.u.

19
mg 1.07 mg/ml