



Date / /

## Cell Passage

ER-5 P1+10 1:4 6-cm dish

1:3 6-well plate CPDL 6h

Hela P1 from Jiyang: in 1ml RNA isoplex (6-cm dish, 100% confluent)

## 6/6 In vitro @ RNA labeling

in vitro

Prepare 2  $\mu$ M MG FAP (NLS-dLS\*) in 25  $\mu$ l DPBS

660nm

$9 \times 60 \times 16 \times 30 = 480 = 4247$   
 $5 \times 33 \times 25 = 825 \times 72 \mu$  (22.9  $\mu$ l, 1.268 mg/ml) + 473  $\mu$ l DPBS.

675nm

Prepare 50  $\mu$ M MG Probes (1  $\mu$ l 0.5 mM + 9  $\mu$ l DMSO)

~~At~~

Prepare 10  $\mu$ g RNA + 2 mM PA in 25  $\mu$ l ~~MF H<sub>2</sub>O~~ DPBS

$333 \times 10 \mu\text{g} = 3330 \mu\text{g RNA}$  (1.901  $\times$  58 + 1.991  $\times$  58 + 1664.8  $\times$  58 + 0.72192  
(825 = 127  $\mu$ l RNA in MF H<sub>2</sub>O) + 698  $\mu$ l DPBS + (8.2  $\mu$ l 2 mM crum prep) PA  $\times 11$

8: MG-H<sub>2</sub>, MG-HI, MG-HBr, <sup>MG-d<sub>2</sub></sup>MG-Br<sub>2</sub>, MG-I<sub>2</sub>, MG-I<sub>2</sub> ester, DMSO

675nm (+/- FAP 8  $\times$  2 = 16

660nm (+/- FAP 8  $\times$  2 = 16

} 32 + 1 = 33

~~34~~

{ 60  $\mu$ l DPBS

① Mix 2.4  $\mu$ l 50  $\mu$ M MG Probes and 60  $\mu$ l 2  $\mu$ M FAP, incubate at 37°C for 10 min { DPBS-MG

② Transfer 60  $\mu$ l MG-FAP to 96-well plate (plate 1).

③ ~~Transfer~~ Plate 2 (675nm): 16 wells, each 25  $\mu$ l (RNA + PA (2mM))

Plate 3 (660nm) = 16 well ~~id~~, each 25  $\mu$ l (RNA + PA (2mM))

④ Transfer 25  $\mu$ l { DPBS-MG  
MG-FAP to 16 wells of plate 2/3.

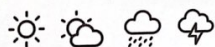
⑤ light irradiation 1 min.

35  $\mu$ l 27  $\mu$ l

⑥ Each well add 400  $\mu$ l RNA isoplex, ~~then 400  $\mu$ l chloroform.~~

Transfer to 1.5 ml tubes (note, mix 675 and 660-DPBS sample)





Date / /

100 $\mu$ l chloroform, 1:1 IPA, with 1 $\mu$ l Glycogen, $-80^{\circ}\text{C}$ , 1 h centrifuge								
resuspend in 20 $\mu$ l NFH <sub>2</sub> O								
conc.	H <sub>2</sub>	HBv	H1	Cl <sub>2</sub>	Bv <sub>2</sub>	I <sub>2</sub>	I <sub>2</sub> ester	DMSO
FAP-675nm	311.80	292.20	315.44	309.92	322.04	295.32	304.96	314.92
FAP-660nm	330.28	315.04	311.32	321.84	339.00	308.56	322.16	319.24
DPBS-675/660nm	604.68	722.72	592.72	650.24	627.00	695.88	646.80	632.88

In vitro

CuAAC (5  $\mu$ g in 26.8  $\mu$ l NFH<sub>2</sub>O)10 min,  $25^{\circ}\text{C}$ , 500 rpm, column purification (~~not~~ reused), 20  $\mu$ l NFH<sub>2</sub>O

6/6 DPBS-675/660nm (3rd reused column for in vivo CuAAC)

7/6 FAP-660nm (2nd reused column for in vivo CuAAC)

7/6 FAP-675nm (3rd  $\checkmark$ )~~H<sub>2</sub> HBv H1~~conc. DMSO H<sub>2</sub> HBv H1 Cl<sub>2</sub> Bv<sub>2</sub> I<sub>2</sub> I<sub>2</sub> ester

FAP-660nm 243.80 247.44 238.80 245.00 237.04 231.88 251.52 241.24

FAP-675nm 244.92 250.80 261.76 246.08 267.92 245.68 269.92 260.16

DPBS-660/675nm 224.04 225.84 219.28 246.52 222.88 219.04 207.92 222.84

Cell labeling

ER-H1

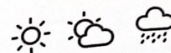
ER-5 P<sub>1</sub>+10 6-well plate

(PA conc.)

125 nM MGHI, 20 min.

PA (nm) 0.25 0.5 1

new prepared 1.5 3 6

res 1  $\mu$ l RAA isopuls.

wash once

Cell

2

7/6. R

R

S

E

(C)

In vitro





Wash once

## Cell Passage

Date

1 1

2022.6.2 new sorted NLS-9

low flow cytometry rate 10-cm dish

In box 3

high flow cytometry rate 10-cm dish = 15 frozen stocks one 1:5 6-cm dish

## 7/6. Cell Passage

ER-5 P1+1 1:5 6-well plate (12h pop)

1:3 6-cm dish

NLS-9 two 6-cm dishes = add 1ml RNA isoplus. save in -80°C

RNA extraction (yesterday labeled), in bowl MF H<sub>2</sub>O

PARMA 0.25 0.5 1 1.5 3 6

conc. 606.60 589.68 675.80 610.28 637.68 554.16

Save 10ug in 26.8ul MF H<sub>2</sub>O aside, in -80°C

Dot CuAAC of in vitro FAP-660nm and FAP-675nm

Dot blot (300ng)

In vitro

FAP-660nm

FAP-675nm

DPBS-660/675nm

DMSO H<sub>2</sub> HBr HI Cl<sub>2</sub> Br<sub>2</sub> I<sub>2</sub> I<sub>2</sub> ester

Dot blot (300ng)

FAP-660nm

FAP-675nm

DPBS-660/675nm

DMSO H<sub>2</sub> HBr HI Br<sub>2</sub> I<sub>2</sub> I<sub>2</sub> ester



Date     /     /

15/6 CuAAC Extract RNA and CuAAC of In-vitro labeled RNA

20ul M <sub>FH2O</sub>	DMSO	H <sub>2</sub>	HBr	H <sub>2</sub>	Cl <sub>2</sub>	Br <sub>2</sub>	I <sub>2</sub>	I <sub>2</sub> ester	
conc.	376.92	367.72	360.00	367.76	360.76	366.44	388.48	383.56	FAP-660
	349.44	331.08	365.56	374.16	352.68	369.88	365.04	348.32	DPBS-660
	527.95	462.70	545.70	442.35	493.15	490.40	488.90	458.40	FAP-675
	359.88	398.24	384.28	373.56	366.16	376.24	380.00	411.32	DPBS-675

in vitro CuAAC: 6.5ug in 20 50 ul System, 25°C, 500rpm, 10 min. column purify (reused)

660/675nm

16ul M <sub>FH2O</sub>	DMSO	H <sub>2</sub>	HBr	H <sub>2</sub>	Cl <sub>2</sub>	Br <sub>2</sub>	I <sub>2</sub>	I <sub>2</sub> ester	
	527.10	529.00	476.60	494.15	520.70	528.25	503.70	505.40	FAP-660
	504.65	494.55	509.85	528.70	545.05	527.25	543.10	517.65	DPBS-660
	404.50	416.65	420.55	424.50	378.95	435.05	420.75	408.90	FAP-675
	526.35	496.35	522.70	514.70	515.25	472.50	523.00	469.70	DPBS-675

16/6 live cell imaging

MS-9 MS-9 Pi+3 500nm MGHB<sub>r</sub>

MS-9 Pi+3 500nm MGHI (cold stock)





Date / /

## 13/5 live cell imaging

1ul hoechst to 2 ml colorless media (pre-heat at 37°C).

H1

One confocal dish (ER-5): remove media, replaced with 1ul 0.5 mM MG-H2 (new) in 1 ml colorless media, incubate 20 min at 37°C. Incubator, then replaced with 1ul hoechst (1/2000), go downstairs to confocal room.

two confocal dishes another plate with +/-

① imaging of confocal dish pre-stained with MG-H2

② time dependent imaging of another dish.

0 min: add 1ul colorless media with 1ul 0.5 mM MG-H2 (new)

result: R100-1.5 turn on and stabilized within 20 min

NLS-9 P1+2: weak signal, also turn on fastly (R100-3)

## 14/5 Cell Passage

FAP-ER-5 P1+3 6cm dish 1:4, 6-well plate (PDL-3h) 1:3

↙ FAP-NLS-5 P1+3 6-cm dish 1:4, 6-well plate (PDL-3h) 1:4  
with 2 mg/ml puromycin

MG-H2 FL turn on kinetics

(5 μM + 5 μM NLS-dLS<sup>xx</sup>) turn on within 1 min and stable during 3h.

## 16/5 Cell passage (14:00)

FAP-ER-5 P1+4 6-cm dish 1:5, 6-well plate (PDL-3h) 1:3

## ER-5 P1+3 labeling

0 125 250

in DMEM

20 min, PA 3 mM, 660 nm 3 min

330ul)

(puromycin)

ER-5

H1





Date / /

MTT Assay: NLS P1+5 ~5% confluency (1:10 200 ul, 22h)

MTT MG-HI (nM) MG-HBr

NLS-9 0, 125, 250, 500, 1000, 2000 125, 250, 500, 1000, 2000

2+6 4+4 2+6 4+4 8ul

Prepare 2 ml MG in DMEM (8ul MG in DMSO)

remove media (set aside)

add 200 ul MG in DMEM (take 8 min)

incubate 20 min, remove MG, refill media, many cells were washed away.

19/5 23h later, remove media, replace with 90ul colorless DMEM + 10ul 5mg/ml MTT.

incubate 3.5h.

remove media, replace with 100ul DMSO, mix by pipetting

result: HBr low toxicity than HI, HI 2000 nM ~ 38%

Ex = 500 or 550 (max) nm

19/5 NLS-9 P1+5 HBr labeling

NLS-9 0 125 250

HBr 500 1000 2000

resuspend in 60 ul M-H<sub>2</sub>O

0 125 250 500 1000 2000

conc. 571.08 543.24 522.28 541.08 526.72 486.92

CuAAC

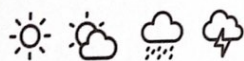
10 ug, p-biotin azide, 25°C, 10 min, 2500 rpm.

column purification (yesterday used), eluted with 20 ul M-H<sub>2</sub>O

0 125 2500 500 1000 2000

conc. 464.96 474.44 483.20 464.56 474.60 470.20





Date / /

Dot Blot (NLS-HBr) 500 ng

0 125 250 500 1000 2000

signal 0.07 1.10 1.05 1.00 1.33 1.43

Agarose Gel 120V, 30 min, 500 ng

→ before loading

0 0 125 250 500 1000 2000

dry loading

use the reused buffer (TAE)

Cell Passage

NLS-9 P1+6 10-cm dish 1:4

two confocal dish 1:5

96-well plate (2x PDL 4h + 2.5x PDL 4h) 1:8, 100ul

counting (24 + 31 + 37 + 31) / 4 = 30.75

1:8, 100ul  $\sim 30.75 \times 2 \times 10^4 \times \frac{1}{8} \times \frac{1}{10} = 0.768 \times 10^4$  cell/well

20/5 ER-5 P1+5 labeling

HBr 0 125 250 HI 0 125 2500

ER-5 500 1000 2000 500 1000 2000

H2, HBr 3mM PA, 660 nm 3 min.

1 ml RNA isoplas in  $-20^{\circ}\text{C}$ , chloroform 200 ul, ~~eluted~~ 70ul NF H<sub>2</sub>O

HBr 0 125 250 500 1000 2000

conc. 503.20 525.52 525.32 522.44 536.36 521.64

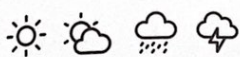
ER-HBr CuAAC 10ug, P-biotin azide, 25°C, 500rpm, 10 min

column purification (3rd time use), eluted with 20ul NF H<sub>2</sub>O

HBr 0 125 250 500 1000 2000

conc. 484.36 474.20 488.08 486.84 482.24 484.80





Date / /

Dot blot (ER-HBr-2) 500ng

0 125 250 500 1000 2000

Signal 0.04 0.44 0.73 1 1.57 1.31

Agarose Gel 120V, 30min, 500ng

0 <sup>before CuAAC</sup>

0 125 250 500 1000 2000

2/15 MTT (ALS, P+6)

NLS-MT1 10:30 remove media, add 100  $\mu$ l MGHR, MGHI  $\Phi$  in DMEM, 20min  
<sup>cells stick on plate well</sup>

Remove media, replace with 100  $\mu$ l fresh media, incubate (11:30 - 11:30)

Remove media, add 90 + 10  $\mu$ l MTT, incubate 3.5h. replaced with  
100  $\mu$ l DMSO.

HBr H1  
0, 125, 200, 250, 500, 1000, 250 500 1000 200 125

live cell imaging

NLS-H1 NLS-9 P+6 confocal dish

500nm MGHI, R200-3.5, em 650 -  $\Phi$  780nm, 0-45min.  
~~AAA~~

NLS RNA Isolation (yesterday labeled H1)

ER-H1 H1 0 125 250 500 1000 2000

conc. 251.72 499.16 521.68 555.72 525.56 552.64

<sup>degraded</sup>

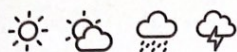
ER-H1 CuAAC = 10ng, P-biotin azide, 25°C, 500rpm, 10min

(0 use the 1st trail, conc. 681.68ng/ $\mu$ l)

H1 0 125 250 500 1000 2000

conc. 448.40 400.04 420.12 425.16 426.84 388.68





Date / /

Dot blot 500 ng

0 125 250 500 1000 2000

Agarose Gel 120V, 30 min, 500 ng

→ before Center

0 0 125 250 500 1000 2000

Cell Passage

NLS-9 P1+7 (from 10-cm dish) (all with 2 kg/ml peno)

(10 ml)

1:5 in 6-cm dish (~1:3)

1:5 in two 6-well plates (~1:3) (PDL 4h)

1:10 in 96 well plate (~1:5) (PDL 4h + overnight)

counting  $(41 + 45 + 56 + 58)/4 = 50$ 

$$(50 \times 2 \times 10^4 \text{ cell/ml}) \times 100 \text{ ul} \times \frac{1}{10} = 1 \times 10^4 \text{ cell/well}$$

ER-5 P1+6 1:10 in 6-cm dish

(5 ml)

1:10 in 96-well plate X3 (PDL 86h)

counting  $(20 + 17 + 21 + 22)/4 = 20$ 

$$(20 \times 2 \times 10^4 \text{ cell/ml}) \times 100 \text{ ul} \times \frac{1}{10} = 0.4 \times 10^4 \text{ cell/well}$$

22/5

MTT Assay

adding take 7 min

NLS NLS-9, 1:10, 24h, 17:30 add MGHBr, MGH2, 100 ul, 20 min

MTT 17:30 replace with fresh media 100 ul

Next day 17:40 replaced with 90 ul colorless DMEM + 10 ul MTT (5 mg/ml)

4h later, replaced with 100 ul DMSO

500 nm, 550 nm Abs.





Date / /

NLS-9 6-well plate (24 h after seeding) Labeling						
NLS-HBr/HI	HBr			HI		
	0	125	250	0	125	250
	500	1000	2000	500	1000	2000
in 1ml RNA isoplus, store in $-80^{\circ}\text{C}$						

23/5 Cell Passage

NLS-9 P1+8

1:5 in 10-cm dish (two) <sup>all</sup> without puromycin

1:10 in 6-cm dish

MTT Assay

ER

ER-5, P1+6 ①, ②, ③ three 96-well plates. (~40%)

MTT

① 17:45, ② 18:08 ③ 18:18 start incubation MTT probe.

After 20 min. replace with media (old 1/2, fresh 1/2)

ER-HBr (1) Dot Blot (ER-HBr, 20% label), 25°C, 10 min, 500 rpm, column purification (new)

0	125	250	500	1000	2000
391.96	389.08				
479.84	420	403.16	429.44	410.44	378.60

Dot blot 500 ng

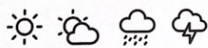
0	125	250	500	1000	2000
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Agarose Gel 120 V, 30 min, 500 ng

→ before GATAC

0	0	125	250	500	1000	2000
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Date / /

	<u>RNA Isolation</u> (Yesterday labeled, <sup>NLS</sup> ER-HBV), 60ul NFHD					
<sup>NLS</sup> ER-HBV	0	125	250	500	1000	2000
conc.	479.84	424.32	444.16	440.00	467.48	480.60

24/5	<u>CuAAC</u> of <sup>NLS</sup> <del>ER</del> -HBV (yesterday isolated)					
<sup>ER</sup> -HBV <sup>NLS</sup>	10 min. p-biotin azide, 25°C, 500 rpm, column purification (2nd)					
	0	125	250	500	1000	2000
conc.	438.44	471.16	472.72	484.68	456.48	452.96

Dot blot 500 ng

0 125 250 500 1000 2000

Agarose Gel 120V, 30 min, 500 ng

→ before CuAAC

0, 0, 125 250 500 1000 2000

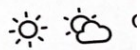
	<u>RNA isolation</u> (yesterday to 22/5 labeled, NLS-H1), 60ul NFHD					
NLS-H1	0	125	250	500	1000	2000
conc.	465.56	453.48	529.56	409.80	517.76	488.48
	Set long in 26.8 ul NFHD aside					

25/5 NLS-9 P47 cell sorting

passage sorted-1 10-cm dish

Sorted-2 10-cm dish contaminated.

	<u>CuAAC</u> of NLS-H1					
NLS-H1	10 min, p-biotin azide, 25°C, 500 rpm, column purification (2nd)					



NLS-H1

26/5

ER/NLS

HBV/H1





Date / /

Dot blot 500 ng  
MS-H1 0 125 250 500 1000 2000

Agarose Gel 120V, 30 min 500 ng

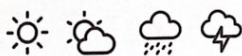
26/5 Dot blot of ER-HBr, ER-H1, MS-HBr, NLS-H1

	500 ng	0	125	250	500	1000	2000
ER/NLS	ER-HBr	429.76	400.72	444.56	507.88	432.52	424.00
HR/H1		1.163	1.248	1.125	0.9845	1.156	1.1792
	ER-H1	445.48	453.38	408.828	434.56	422.60	454.32
		1.1224	1.1028	1.2246	1.1506	1.1832	1.1005
	MS-HBr	464.96	474.44	483.20	464.56	474.60	470.20
	<del>MS-H1</del>	1.0754	1.0539	1.0348	1.0763	1.0535	1.0634
	NLS-H1	485.60	489.56	468.72	474.24	477.72	490.08
		1.0296	1.0213	1.0667	1.054	1.0466	1.020

Cell Passage  
10-cm dish + 6-cm dish NLS-9 P1+8 in 4 ml RNAi supernatant  
to four 1.5 ml tubes, in -80°C

2/5 Cell Passage  
MS-9 P1+1 from frozen stock  
1:5 in 6-cm dish





Date / /

Cell labeling ER P+8 6-well plates

ER-H1

MGH2 125<sub>nM</sub> ( PA 1.5, 3, 6 mM)0<sub>nM</sub> ( PA 1.5, 3, 6 mM) PA

light 1 min or 2 min

↓ PA 2mM stock → PA 1mM stock

1ml RNA 750plus, 60ul MF H<sub>2</sub>O

125-1.5-1 468.48

125-1.5-2

125-3-1 480.12

125-3-2 476.12

125-6-1 515.68

125-6-2 560.92

0-1.5-1 518.80

0-1.5-2 488.56

0-3-1 544.76

0-3-2 636.84

0-6-1 504.64

0-6-2 510.48

547.04

ER-H1

CuAAC: 210<sub>ng</sub>, P-biotin azide, 10 min, 25°C, 500 rpm.column purification (3rd, 4th, (H<sub>2</sub>O))

125-1.5-1 399.80

125-1.5-2 489.96

125-3-1 461.64

125-3-2 487.16

125-6-1 471.80

125-6-2 499.64

0-1.5-1 462.44

0-1.5-2 511.72

0-3-1 473.80

0-3-2 499.36

0-6-1 462.88

0-6-2 483.20

ER-H1

Dot blot 500<sub>ng</sub> similar

double compare to 1 min

125-1.5-1

125-3-1

125-6-1

125-1.5-2

125-3-2

125-6-2

0-1.5-1

0-3-1

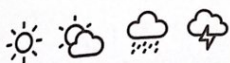
0-6-1

0-1.5-2

0-3-2

0-6-2





Date / /

Agarose Gel 50mg 120V, 30 min.

(1) 125-1.5-100, 125-3-1, 125-6-1, 0-1.5-1, 0-0.3-1, 0-6-1

(2) --- -2 -2 -2 -2 -2 -2

28/5 Cell Passage

ER-5 P1+8

(~1 day) 1:4 6-cm dish

1:10 6-cm dish

(~4 days) 1:10 two confocal dish (PDL 5h)

(~1 x day) 1:4 6-well plate (PDL 5h)

29/5 ADPA bleaching

160ul 5uM FAP-5uM MG (45h incubation), 675 nm, 330 W/m<sup>2</sup>, 0, 2, 4, 6, 8 min

PL, PE, UV-Vis of (5uM FAP-5uM MG) x  $\frac{12}{10}$

30/5 Cell labeling

ER-H1 ER P1+8 6-well plate, HI 125 nM, 0.25/0.5/1 mM PA, light 1 min

PA 1 0.25 0.5

1 0.25 0.5

1 ml RNA isoplas, 70 ul MFH20

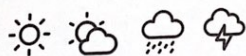
conc. HI 0.25 0.5 0.1

conc. 454.20 496.64 388.20

DMSO 0.25 0.5 1

conc. 436.00 446.32 400.48





Date / /

CuAAC ER-H1, 10 min, 25°C, 500 rpm, column purification (new)

ER-H1	MgH1	0.25	0.5	1
	conc.	424.12	426.92	427.20
	DMSO	0.25	0.5	1
	conc.	423.16	424.16	426.44

31/5 Dot blot (500 ng)

ER-H1 test 27/5 RNA sample <sup>conc.</sup> again

MgH1	1.5	3	6
	391.04	455.20	467.31
DMSO	1.5	3	6
	456.88	405.68	455.20

MgH1	0.25	0.5	1	1.5	3	6
DMSO	0.25	0.5	1	1.5	3	6

Agarose Gel (500 ng), 120V, 30 min

0.25	0.5	1	0.25	0.5	1	1 kb (m)
MgH1			DMSO			

live cell imaging after PA labeling

ER-H1





Date / /

30/5

Cell Passage

NLS-9 P12 1:10 10-cm dish

1:10 6-cm dish

ER-5 P19 1:8 96-well plates X3 for MTT (PDL 6h)

1:4 6-cm dishes X2 (PDL 6h)

30/5

1/6

MTT Assay

MTT-

~60% confluency, MGd2. 20 min

ER-Cl2

125, 250, 500, 1000, 2000, 0, empty

3/6 24h later, replace with 90ul colorless media + 10ul MTT

3.5h later replace with 100ul DMSO, 500/550 nm OD

2/6

ER-5 P19 Cell Labeling

6-cm dishes: 125 nM MGHI or 0 nM MGHI

ER-HI

(PA conc.)

1 mM PA; light 1 min.

RNA ex 1 ml RNA isoplas, 100 ul MFH2O

125-1-1 0-1-1

1000. 537.12 507.20

4 tubes: 26.8 ul, 10 ug

Total RNA Isolation (5 tubes of cell lysate in 1 ml RNA isoplas, MS-9), 60 ul

Total RNA 1 2 3 4 5

combine (2)

combine (3)

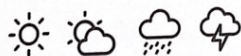
1991.0

1901.5

1664.8

&gt;15 ug/75 ul





Date / /

ER-5 P1+9 6-well plate labeling

ER-H1 MG H1 0.75% 0.5% 1%  
(125nm)  
(PA conc) DMSO 0.75% 0.5% 1% , light 1 min

1ml RNA Isoplus, 60ul MF-H<sub>2</sub>O

Cell Passage

MS-9 P1+4 1:20 in 6-cm dish

MS-9 (P1+4) sorted today

① high cell number in 10-cm dish

② low cell number in 6-cm dish

4/6

CuAAC of ER-H125°C, 10 min / <sup>15</sup>min / 20 min / 25 min, 500 rpm, 100ng

ER-H1

new column purification

(CuAAC Time)

conc. 10 min { MG H1 CP } 312.8

{ DMSO (N) } 344.28

15 min { P } 348.20

{ N } 312.04

20 min { P } 337.00

{ N } 345.64

25 min { P } 364.28

{ N } 335.16

Dot blot 500ng

result: 10-25 min similar intensity

→ <sup>15</sup>min - 25 min slightly higher background than 10 min

Agarose Gel: 500ng, 120V, 30min

TAE PAGE Gel: 500ng, 120V, 70 min: similar integrity.





Date / /

## 8/6 Cell Passage

MS-9 new sorte (high) 1:20 6-cm dish

1:3 (500  $\mu$ l) confocal dish.

MS-9 new sorte (low) 1:10 6-cm dish

1:3 (500  $\mu$ l) two confocal dishes1:6 (250  $\mu$ l) two confocal dishesCuAAC (ER-5 H1 175 nm)

ER-H1 PA 0.25, 0.5, 1, 1.5, 3, 6 mM

PA conc. 10  $\mu$ g, 25°C, 500 rpm, 10 min, @ reusol column purification, 20  $\mu$ l NF H<sub>2</sub>O

PA (mM) 0.25 0.5 1 1.5 3 6

conc. 467.52 453.92 467.08 451.60 458.28 378.12

Dot blot 500 ng

Agarose Gel 100 ng, 120V, 30 min

Cell labeling

ER-H1 ER P1+11 0 mM MG H1, PA 0.25, 0.5, 1, 1.5, 3, 6 mM

PA conc. 1 ml RNA isoplu

9/6 RNA extraction, 50  $\mu$ l NF H<sub>2</sub>O

0 mM MG H1 PA (mM) 0.25 0.5 1 1.5 3 6

conc. 485.48 502.40 499.84 503.20 489.60 467.24

CuAAC (ER-5 H1 0 mM)10  $\mu$ g, 25°C, 500 rpm, 10 min, new column purification, 20  $\mu$ l NF H<sub>2</sub>O

0 mM MG H1 PA (mM) 0.25 0.5 1 1.5 3 6

conc. 433.64





Date / /

Dot blot (500 ng)

PA (mM) 0.25 0.5 1 1.5 3 6

MGH1 125 nM

MGH1 0 nM

Agarose Gel (500 ng)

MGH1 0 nM PA 0.25 0.5 1 1.5 3 6

Live cell imaging = MGHBr 500 nM, NLS-9 new sorting (low) 0-30 min. (and high)

10/6 Cell labeling ER Pi+12 (signal outside nucleus)

125 nM MGH1, PA 0.25, 0.5, 1, 1.5, 3, 6 mM.

1 ml RNA isoprep, 60 ul MFH<sub>2</sub>O

0.25 0.5 1 1.5 3 6

conc 658.72 673.24 662.48 625.48 636.48 633.24

Live cell imaging

NLS-9 new sorting (low), MGH1 500 nM, 0-30 min

Cell Passage

new stock NLS-9 Pi 6-cm dish

11/6 CwAAC (ER MGH1 125 nM)

100 ng, 10 min, 25°C, 500 rpm, 20 ul MFH<sub>2</sub>O

0.25 0.5 1 1.5 3 6

conc. 451.28 494.28 503.44 483.22 503.36 534.12

Cell Passage Dot blot: 500 ng

Agarose Gel: 500 ng





Date / /

20/6

MTT Assay

ER-5 P116 (~70% confluency)

96-well plates

ERH1, hv

① No MG, ② MGHI 125 nM, ③ MGHI 125 nM + 1 mM PA

light 1 min, wash twice with DPBS. replace with media

plate 1: 4h later, 981ul colorless media + 9ul MTT, 3.5h

plate 2: 24h later, - - -

Cell labeling

ER-5 P115 ~80% confluency 10-cm dishes

ER-H1

 $\left\{ \begin{array}{l} \text{MGHI 125 nM (3ml)} \\ \text{DMSO (3ml)} \end{array} \right. 20 \text{ min.}$ 

PA 1 mM (newly prepared), 660 nm light 1 min.

① 3 ml RNA isoplus, divided to 3 tubes, resuspend in 60 ul RFLN

MGHI (125-1-1)

DMSO (0-1-1)

① 1097.4

① 1148.8

② 1046.2

② 1216.4

③ 967.92

③ 1142.7

Mix ①, ②, ③ and re-divided to 3 tubes. Save in -80°C

① 5/2.5uM 20 min - 10 - 10 min

② 5uM 20 min - 30 - 10 min

③ 2.5/5uM 15 min - 10 - 10 min

Cell imaging - labeling

hoe-E63-DPF in 5% DMSO, 1 ml HBSS (2.5 uM or 5 uM or 0 uM)

incubate 20 min or 15 min, rinse with 1 ml HBSS

incubate 1 ml media, twice (30 + 10 min or 10 + 10 min)

2 mM PA, Green light 1 min (Note: (5/2.5uM 15-10-10 min not enough light exposure)) signal is lower than the other three

~~Go to~~ PFA 15 min, 0.1% Triton 5 min, wash twice, blocking 1h, wash twice, ~~Go to~~ 1h, wash (0.1% Triton) X3, DPBS X1, hoechst 3 min, DPBS X1

Result: negative show TAMRA background (spots), all show good localization





Date / /

21/6

DNase + PK

ER-H1

yesterday isolated RNA (ER-H1)

③ 125-1-1, 59  $\mu$ l, ~1000 ng/ $\mu$ l      ③ 0-1-1, 59  $\mu$ l, ~1100 ng/ $\mu$ lAdd 41  $\mu$ l NFH<sub>2</sub>O, 11.5  $\mu$ l 10X DNase<sup>latter</sup> (Turbo), 3  $\mu$ l Turbo DNase, 37°C,  
PK 2  $\mu$ l, 42°C, 15 min, 300 rpm  
300-rpm, 15 min. New column purification, eluted with 40 + 63  $\mu$ l NFH<sub>2</sub>O  
(some white solid (column matrix))

③ 125-1-1 - DNase

③ 0-1-1 - DNase

conc. 455.84

555.80

ER-H1

CuAAC(20  $\mu$ g in 100  $\mu$ l Reaction) X2, 25°C, 500 rpm, 10 mincolumn purification (reuse the DNase-PK column), eluted with ~~50~~<sup>40</sup> + 62  $\mu$ l NFH<sub>2</sub>O

③ 125-1-1 CuAAC

③ 0-1-1 CuAAC

conc. 410.32

372.28

Set 3 tubes of 10  $\mu$ g RNA in 50  $\mu$ l NFH<sub>2</sub>O aside (-80°C)

Dot blot (500 ng)

- CuAAC	MGH1	DMSO
+ CuAAC	MGH1	DMSO

Agarose Gel (500 ng)

MGH1	DMSO	MGH1	DMSO	1 kb
- CuAAC		+ CuAAC		

22/6

Cell Passage

HEK 293T (from Jinhua) P18 1:6 in three 96-well plates (PDL overnight)

ER-5 P17 1:20 in 6-cm dish

(~100%)

1:8 in 96-well plate (PDL overnight)

MS-9 P15 1:30 in 6-cm dish

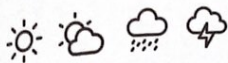
(100%)

remain  
pellet in -80°C

MS-9 P16 (new sort, high) 1:30 in 6-cm dish

(100%)





Date / /

## Enrichment

Yesterday 7- MGHI / ER 10ug RNA, CI beads 20ul, 1.5h at 25°C  
 ER-H1 (Mix Mode, 18 rate)

Eluted with 10ul MFH<sub>2</sub>O (recover ~ 8.5ul)

Dilute RNA before enrichment to ~  $\frac{100}{200}$  ng/ml (2ug in 20ul)

Qubit 4 CHS)

	+ MGHI(I <sub>n</sub> )	- MGHI(I <sub>n</sub> )	+ MGHI(en)	- MGHI(en)	S <sub>1</sub>	S <sub>2</sub>
conc (1ul)	118	116	too low	too low	51.80	800.63
(2ul)			too low	too low		(500 ng/ml)

(Broad)

					49.87	766.64
	too high	too high	17.2	too low	too low	978
			2ul $\frac{1.758 \text{ ng}}{200 \text{ ul}}$ (0.88 ng/ml)			$\frac{100 \text{ ng}}{200 \text{ ul}}$

$$\frac{0.88 \times 8.5 \text{ ul}}{10 \times 10^3 \text{ ng}} \times 100\% = 7.48 \times 10^{-2}\% \text{ (too low recovery rate!)}$$

## Flow through

Add 200ul MFH<sub>2</sub>O, 600ul Binding <sup>buffer</sup> for column purification (20ul MFH<sub>2</sub>O)  
 + MGHI(FT) - MGHI(FT)

conc.	398.24	418.40
-------	--------	--------

## Dot blot

500ng for input and flow-through, 0.5ul for enriched RNA

input	+	(-)
enrich	+	-
flow-through	(+)	-

Similar level





Date

/ /

23/6

MTT Assay (Dark)

HEK-MG

HEK 293T 178 (0-2000 nM MGHBV, MGHI8, 20 min), replace with  
 24h later (10:30-11:10), replace with 90+10ul MTT, 3.5h.

recycled  
 ↑  
 media

MTT Assay (light)

DBF-ER

ER-5 P1+17

0um DBF, 2.5um DBF, 2.5um DBF + 2mM PA, light 1 min.

DBF incubation: remove media, 100ul HBSS rinse, 40ul DBF/HBSS 15 min.

then 100ul HBSS rinse, wash twice with media 100ul, 10 min each time.

replace with media <sup>→ recycled</sup> (a lot of cells are washed away)

24h later (12:00), replace with 90+10ul MTT, 3.5h.

Enrichment

ER-H1

2x4 = 84ul Cl beads +/- ER-H1 10ug RNA, 20ul beads, 1.5hat20c

A +/- MG-H1 DPBS wash, 60c 5min + 90c 5min (50ul) ⇒ 50ul (intotal)

B +/- MG-H1 60c 5min (50ul), (new 20ul) 90c 5min ⇒ 70ul (intotal)

(bit 4 (HS) (1ul)

	A+	A-	B+	B-	S1	S2
(HS) conc.	too low	too low	too low	too low	52.58	829.23
(Bract)	25.8	too low	14.8	too low	51.61	828.94
real conc.	$\frac{3.11}{200ul}$		$\frac{1.785}{200ug}$			$\frac{100ug}{200ul}$

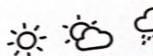
$$\frac{3.11 ng/ml \times 8.5ul}{10 \times 10^3 ng} = 0.26\%$$

$$\frac{1.785 \times 8.5}{10 \times 10^3 ng} = 0.15\%$$

yesterday 0.075% @

Flow through: 150ul NFIH20, column purification, eluted with 20ul NFIH20

con. A+ 518.84 A- 111 B+ D-



24/6

Cl





Date / /

## Cell Passage

ER-5 P1+10 1:4 6-cm dish

1:3 6-well plate CPDL 6h

Hela P1 from Jiyang: in 1ml RNA isoplex (6-cm dish, 100% confluent)

## 6/6 In vitro @ RNA labeling

in vitro

Prepare 2  $\mu$ M MG FAP (NLS-dLS\*) in 25  $\mu$ l DPBS

660nm

$9 \times 60 \times 16 \times 30 = 4320$   
 $5 \times 33 \times 25 = 825$   
 $473$   
 $22.9 \mu$ l, 1.268 mg/ml + 473  $\mu$ l DPBS.

675nm

Prepare 50  $\mu$ M MG Probes (1  $\mu$ l 0.5 mM + 9  $\mu$ l DMSO)

~~At~~

Prepare 10  $\mu$ g RNA + 2 mM PA in 25  $\mu$ l ~~MF H<sub>2</sub>O~~ DPBS

$333 \times 10 \mu\text{g} = 3330 \mu\text{g RNA}$  (1.901  $\times$  58 + 1.991  $\times$  58 + 1664.8  $\times$  58 + 0.72192  
(825 = 127  $\mu$ l RNA in MF H<sub>2</sub>O) + 698  $\mu$ l DPBS + (8.2  $\mu$ l 2 mM <sup>MG-dL<sub>2</sub></sup> ~~crum prep~~) PA <sup>x11</sup>

8: MG-H<sub>2</sub>, MG-H<sub>1</sub>, MG-HBr, <sup>MG-dL<sub>2</sub></sup> MG-Br<sub>2</sub>, MG-I<sub>2</sub>, MG-I<sub>2</sub> ester, DMSO

675nm (+/- FAP 8  $\times$  2 = 16

660nm (+/- FAP 8  $\times$  2 = 16

} 32 + 1 = 33

~~34~~

{ 60  $\mu$ l DPBS

① Mix 2.4  $\mu$ l 50  $\mu$ M MG Probes and 60  $\mu$ l 2  $\mu$ M FAP, incubate at 37°C for 10 min { DPBS-MG

② Transfer 60  $\mu$ l MG-FAP to 96-well plate (plate 1).

③ ~~Transfer~~ Plate 2 (675nm): 16 wells, each 25  $\mu$ l (RNA + PA (2mM))

Plate 3 (660nm) = 16 well ~~id~~, each 25  $\mu$ l (RNA + PA (2mM))

④ Transfer 25  $\mu$ l { DPBS-MG  
MG-FAP to 16 wells of plate 2/3.

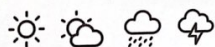
⑤ light irradiation 1 min.

35  $\mu$ l 27  $\mu$ l

⑥ Each well add 400  $\mu$ l RNA isoplex, ~~then 400  $\mu$ l chloroform.~~

Transfer to 1.5 ml tubes (note, mix 675 and 660-DPBS sample)





Date / /

100 ul chloroform, 1:1 IPA, with 1 ul Glycogen, -80°C, 1 h centrifuge								
resuspend in 20 ul NFH <sub>2</sub> O								
conc.	H <sub>2</sub>	HBv	H1	Cl <sub>2</sub>	Bv <sub>2</sub>	I <sub>2</sub>	I <sub>2</sub> ester	DMSO
FAP-675 nm	311.80	292.20	315.44	309.92	322.04	295.32	304.96	314.92
FAP-660 nm	330.28	315.04	311.32	321.84	339.00	308.56	322.16	319.24
DPBS-675/660 nm	604.68	722.72	592.72	650.24	627.00	695.88	646.80	632.88

In vitro

CuAAC (5 ug in 26.8 ul NFH<sub>2</sub>O)10 min, 25°C, 500 rpm, column purification (not reused), 20 ul NFH<sub>2</sub>O

6/6 DPBS-675/660 nm (3rd reused column for in vivo CuAAC)

7/6 FAP-660 nm (2nd reused column for in vivo CuAAC)

7/6 FAP-675 nm (3rd reused)

~~H<sub>2</sub> HBv H1~~conc. DMSO H<sub>2</sub> HBv H1 Cl<sub>2</sub> Bv<sub>2</sub> I<sub>2</sub> I<sub>2</sub> ester

FAP-660 nm 243.80 247.44 238.80 245.00 237.04 231.88 251.52 241.24

FAP-675 nm 244.92 250.80 261.76 246.08 267.92 245.68 269.92 260.16

DPBS-660/675 nm 224.04 225.84 219.28 246.52 222.88 219.04 207.92 222.84

Cell labeling

ER-H1

ER-5 P<sub>1</sub>+10 6-well plate

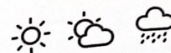
(PA conc.)

125 nM MGHI, 20 min.

PA (nm) 0.25 0.5 1

new prepared 1.5 3 6

res 1 ml RAA isopuls.



Cell

wash once

2

7/6. R

R

S

In vitro





Wash once

## Cell Passage

Date

/ /

2022.6.2 new sorted NLS-9

In box 3

low flow cytometry rate 10-cm dish

high flow cytometry rate 10-cm dish = 15 frozen stocks

one 1:5 6-cm dish  
one 1:5 6-cm dish

## 7/6. Cell Passage

ER-5 P1+1 1:5 6-well plate (12h pop)  
1:3 6-cm dish

NLS-9 two 6-cm dishes = add 1ml RNA isoplus. save in -80°C

RNA extraction (yesterday labeled), in 60ul MF H<sub>2</sub>O

PARMA 0.25 0.5 1 1.5 3 6

conc. 606.60 589.68 675.80 610.28 637.68 554.16

Save 10ul in 26.8ul MF H<sub>2</sub>O aside, in -80°C

Dot CNAAC of in vitro FAP-660nm and FAP-675nm

Dot blot (300ng)

In vitro

FAP-660nm

FAP-675nm

DPBS-660/675nm

DMSO H<sub>2</sub> HBr HI Cl<sub>2</sub> Br<sub>2</sub> I<sub>2</sub> I<sub>2</sub> ester

Dot blot (300ng)

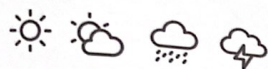
FAP-660nm

FAP-675nm

DPBS-660/675nm

DMSO H<sub>2</sub> HBr HI Br<sub>2</sub> I<sub>2</sub> I<sub>2</sub> ester





Date     /     /

15/6 CuAAC Extract RNA and CuAAC of In-vitro labeled RNA

20ul M <sub>FH2O</sub>	DMSO	H <sub>2</sub>	HBr	H <sub>2</sub>	Cl <sub>2</sub>	Br <sub>2</sub>	I <sub>2</sub>	I <sub>2</sub> ester	
conc.	376.92	367.72	360.00	367.76	360.76	366.44	388.48	383.56	FAP-660
	349.44	331.08	365.56	374.16	352.68	369.88	365.04	348.32	DPBS-660
	527.95	462.70	545.70	442.35	493.15	490.40	488.90	458.40	FAP-675
	359.88	398.24	384.28	373.56	366.16	376.24	380.00	411.32	DPBS-675

in vitro CuAAC: 6.5ug in 20 50 ul System, 25°C, 500rpm, 10 min. column purify (reused)

660/675nm

16ul M <sub>FH2O</sub>	DMSO	H <sub>2</sub>	HBr	H <sub>2</sub>	Cl <sub>2</sub>	Br <sub>2</sub>	I <sub>2</sub>	I <sub>2</sub> ester	
	527.10	529.00	476.60	494.15	520.70	528.25	503.70	505.40	FAP-660
	504.65	494.55	509.85	528.70	545.05	527.25	543.10	517.65	DPBS-660
	404.50	416.65	420.55	424.50	378.95	435.05	424.75	408.90	FAP-675
	526.35	496.35	522.70	514.70	515.25	472.50	523.00	469.70	DPBS-675

16/6 live cell imaging

MS-9 NLS-9 P1+3 500nm MGHB<sub>r</sub>

NLS-9 P1+3 500nm MGHI (cold stock)





Date / /

## 13/5 live cell imaging

1ul hoechst to 2 ml colorless media (pre-heat at 37°C).

H1

One confocal dish (ER-5): remove media, replaced with 1ul 0.5 mM MG-H2 (new) in 1 ml colorless media, incubate 20 min at 37°C. Incubator, then replaced with 1ml hoechst (1/2000), go downstairs to confocal room.

two confocal dishes another plate with +/-.

① imaging of confocal dish pre-stained with MG-H2

② time dependent imaging of another dish.

0 min: add 1ul colorless media with 1ul 0.5 mM MG-H2 (new)

result: R100-1.5 turn on and stabilized within 20 min

NLS-9 P1+2: weak signal, also turn on fastly (R100-3)

## 14/5 Cell Passage

FAP-ER-5 P1+3 6cm dish 1:4, 6-well plate (PDL-3h) 1:3

↙ FAP-NLS-5 P1+3 6-cm dish 1:4, 6-well plate (PDL-3h) 1:4  
with 2mg/ml puromycin

MG-H2 FL turn on kinetics

(5uM + 5uM NLS-dLS\*\*) turn on within 1min and stable during 3h.

## 16/5 Cell passage (14:00)

FAP-ER-5 P1+4 6-cm dish 1:5, 6-well plate (PDL-3h) 1:3

330ul)

(puromycin)

ER-5

H1

## ER-5 P1+3 labeling

0 125 250

in DMEM

PA 3 mM, 660 nm 3 min  
20 min





Date / /

MTT Assay: NLS P1+5 ~5% confluency (1:10 200 ul, 22h)

MTT MG-HI (nM) MG-HBr

NLS-9 0, 125, 250, 500, 1000, 2000 125, 250, 500, 1000, 2000

2+6 4+4 2+6 4+4 8ul  
Prepare 2 ml MG in DMEM (8ul MG in DMSO)

remove media (set aside)

add 200 ul MG in DMEM (take 8 min)

incubate 20 min, remove MG, refill media

many cells were washed away.

19/5 23h later, remove media, replace with 90ul colorless DMEM + 10ul 5mg/ml MTT.

incubate 3.5h.

remove media, replace with 100ul DMSO, mix by pipetting

result: HBr low toxicity than HI, HI 2000 nM ~ 38%

Ex = 500 or 550 (max) nm

19/5 NLS-9 P1+5 HBr labeling

NLS-9 0 125 250

HBr 500 1000 2000

resuspend in 60 ul M-H<sub>2</sub>O

0 125 250 500 1000 2000

conc. 571.08 543.24 522.28 541.08 526.72 486.92

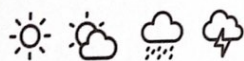
CuAAC

10 ug, p-biotin azide, 25°C, 10 min, 2500 rpm.

column purification (yesterday used), eluted with 20 ul M-H<sub>2</sub>O

0 125 2500 500 1000 2000  
conc. 464.96 474.44 483.20 464.56 474.60 470.20





Date / /

Dot blot (NLS-HBr) 500 ng

0 125 250 500 1000 2000

signal 0.07 1.10 1.05 1.00 1.33 1.43

Agarose Gel 120V, 30 min, 500 ng

→ before loading

0 0 125 250 500 1000 2000

dry loading

use the reused buffer (TAE)

Cell Passage

NLS-9 P1+6 10-cm dish 1:4

two confocal dish 1:5

96-well plate (2x PDL 4h + 2.5x PDL 4h) 1:8, 100ul

counting (24 + 31 + 37 + 31) / 4 = 30.75

1:8, 100ul  $\sim 30.75 \times 2 \times 10^4 \times \frac{1}{8} \times \frac{1}{10} = 0.768 \times 10^4$  cell/well

20/5 ER-5 P1+5 labeling

HBr 0 125 250 HI 0 125 2500

ER-5 500 1000 2000 500 1000 2000

H2, HBr 3mM PA, 660 nm 3 min.

1 ml RNA isoplas in  $-20^{\circ}\text{C}$ , chloroform 200 ul, ~~eluted~~ 70ul NF H<sub>2</sub>O

HBr 0 125 250 500 1000 2000

conc. 503.20 525.52 525.32 522.44 536.36 521.64

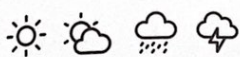
ER-HBr CuAAC 10ug, P-biotin azide, 25°C, 500rpm, 10 min

column purification (3rd time use), eluted with 20ul NF H<sub>2</sub>O

HBr 0 125 250 500 1000 2000

conc. 484.36 474.20 488.08 486.84 482.24 484.80





Date / /

Dot blot (ER-HBr-2) 500ng

0 125 250 500 1000 2000

Signal 0.04 0.44 0.73 1 1.57 1.31

Agarose Gel 120V, 30min, 500ng

0 <sup>before CuAAC</sup>

0 125 250 500 1000 2000

2/15 MTT (ALS, P+6)

NLS-MT1 10:30 remove media, add 100  $\mu$ l MGHR, MGHI  $\Phi$  in DMEM, 20min  
<sup>cells stick on plate well</sup>

Remove media, replace with 100  $\mu$ l fresh media, incubate (11:30 - 11:30)

Remove media, add 90 + 10  $\mu$ l MTT, incubate 3.5h, replaced with  
100  $\mu$ l DMSO.

HBr

HI

0, 125, 2000, 250, 500, 1000, 250 500 1000 200 125

Live cell imaging

NLS-H1 NLS-9 P+6 confocal dish

500 nm MGHI, R200-3.5, em 650 -  $\Phi$  780 nm, 0 - 45 min.  
~~AAA~~

NLS RNA Isolation (yesterday labeled HI)

ER-H1 HI 0 125 250 500 1000 2000

conc. 251.72 499.16 521.68 555.72 525.56 552.64

degraded

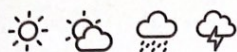
ER-H1 CuAAC: 10 ng, P-biotin azide, 25°C, 500 rpm, 10min

(0 use the 1st trail, conc. 681.68 ng/ $\mu$ l)

HI 0 125 250 500 1000 2000

conc. 448.40 400.04 420.12 425.16 426.84 388.68





Date / /

Dot blot 500 ng

0 125 250 500 1000 2000

Agarose Gel 120V, 30 min, 500 ng

→ before Center

0 0 125 250 500 1000 2000

Cell Passage

NLS-9 P1+7 (from 10-cm dish) (all with 2 kg/ml peno)

(10 ml)

1:5 in 6-cm dish (~1:3)

1:5 in two 6-well plates (~1:3) (PDL 4h)

1:10 in 96 well plate (~1:5) (PDL 4h + overnight)

counting  $(41 + 45 + 56 + 58)/4 = 50$ 

$$(50 \times 2 \times 10^4 \text{ cell/ml}) \times 100 \text{ ul} \times \frac{1}{10} = 1 \times 10^4 \text{ cell/well}$$

ER-5 P1+6 1:10 in 6-cm dish

(5 ml)

1:10 in 96-well plate X3 (PDL 86h)

counting  $(20 + 17 + 21 + 22)/4 = 20$ 

$$(20 \times 2 \times 10^4 \text{ cell/ml}) \times 100 \text{ ul} \times \frac{1}{10} = 0.4 \times 10^4 \text{ cell/well}$$

22/5

MTT Assay

adding take 7 min

NLS NLS-9, 1:10, 24h, 17:30 add MGHBv, MGHI, 100 ul, 20 min

MTT 17:30 replace with fresh media 100 ul

Next day 17:40 replaced with 90 ul colorless DMEM + 10 ul MTT (5 mg/ml)

4h later, replaced with 100 ul DMSO

500 nm, 550 nm Abs.





Date / /

NLS-9 6-well plate (24 h after seeding) <u>Labeling</u>						
NLS-HBr/HI	HBr			HI		
	0	125	250	0	125	250
	500	1000	2000	500	1000	2000
in 1ml RNA isoplus, store in $-80^{\circ}\text{C}$						

23/5 Cell Passage

NLS-9 P1+8

1:5 in 10-cm dish (two) <sup>all</sup> without puromycin

1:10 in 6-cm dish

MTT Assay

ER

ER-5, P1+6 ①, ②, ③ three 96-well plates. (~40%)

MTT

① 17:45, ② 18:08 ③ 18:18 start incubation MTT probe.

After 20 min. replace with media (old  $\frac{1}{2}$ , fresh  $\frac{1}{2}$ )ER-HBr (1) Dot Blot (ER-HBr, 20% label),  $25^{\circ}\text{C}$ , 10 min, 500 rpm, column purification (new)

0	125	250	500	1000	2000
391.96	389.08				
479.84	420	403.16	429.44	410.44	378.60

Dot blot 500 ng

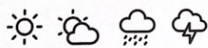
0	125	250	500	1000	2000
---	-----	-----	-----	------	------

Agarose Gel 120 V, 30 min, 500 ng

→ before GAAc

0	0	125	250	500	1000	2000
---	---	-----	-----	-----	------	------





Date / /

	<u>RNA Isolation</u> (Yesterday labeled, <sup>NLS</sup> ER-HBv), 60ml NPHD					
ER-HBv NLS	0	125	250	500	1000	2000
conc.	479.84	424.32	444.16	440.00	467.48	480.60

24/5	<u>CNAAC</u> of <sup>NLS</sup> ER-HBv (yesterday isolated)					
ER-HBv NLS	10 min. p-biotin azide, 25°C, 500 rpm, column purification (2nd)					
	0	125	250	500	1000	2000
conc.	438.44	471.16	472.72	484.68	456.48	452.96

Dot blot 500 ng

0 125 250 500 1000 2000

Agarose Gel 120V, 30 min, 500 ng

→ before CNAAC

0, 0, 125 250 500 1000 2000

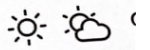
	<u>RNA Isolation</u> (yesterday to 22/5 labeled, NLS-H1), 60ml NPHD					
NLS-H1	0	125	250	500	1000	2000
conc.	465.56	453.48	529.56	409.80	517.76	488.48
Set long in 26.8 ml NPHD aside						

25/5 NLS-9 P47 cell sorting

passage sorted-1 10-cm dish

Sorted-2 10-cm dish contaminated.

	<u>CNAAC</u> of NLS-H1					
NLS-H1	10 min, p-biotin azide, 25°C, 500 rpm, column purification (2nd)					



NLS-H1

26/5

ER/NLS

HBv/H1





Date / /

Dot blot 500 ng  
MS-H1 0 125 250 500 1000 2000

Agarose Gel 120V, 30 min 500 ng

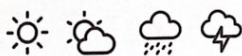
26/5 Dot blot of ER-HBr, ER-H1, MS-HBr, NLS-H1

	500 ng	0	125	250	500	1000	2000
ER/NLS	ER-HBr	429.76	400.72	444.56	507.88	432.52	424.00
HR/H1		1.163	1.248	1.125	0.9845	1.156	1.1792
	ER-H1	445.48	453.38	408.828	434.56	422.60	454.32
		1.1224	1.1028	1.2246	1.1506	1.1832	1.1005
	MS-HBr	464.96	474.44	483.20	464.56	474.60	470.20
	<del>MS-H1</del>	1.0754	1.0539	1.0348	1.0763	1.0535	1.0634
	NLS-H1	485.60	489.56	468.72	474.24	477.72	490.08
		1.0296	1.0213	1.0667	1.054	1.0466	1.020

Cell Passage  
10-cm dish + 6-cm dish NLS-9 P1+8 in 4 ml RNAi supernatant  
to four 1.5 ml tubes, in -80°C

2/5 Cell Passage  
MS-9 P1+1 from frozen stock  
1:5 in 6-cm dish





Date / /

## Cell labeling ER P+8 6-well plates

ER-H1

MuH2 125<sub>nM</sub> ( PA 1.5, 3, 6 mM)

0<sub>nM</sub> ( PA 1.5, 3, 6 mM) PA

light 1 min or 2 min

↓ PA 2mM stock → PA 1mM stock

1ml RNA 750plus, 600ul MF H<sub>2</sub>O

125-1.5-1 468.48

125-1.5-2

125-3-1 480.12

125-3-2 476.12

125-6-1 515.68

125-6-2 560.92

0-1.5-1 518.80

0-1.5-2 488.56

0-3-1 544.76

0-3-2 636.84

0-6-1 504.64

0-6-2 510.48

547.04

ER-H1

CuAAC: <sup>210</sup>ug, P-biotin azide, 10 min, 25°C, 500 rpm.

column purification (3rd, 4th, (H<sub>2</sub>O))

125-1.5-1 399.80

125-1.5-2 489.96

125-3-1 461.64

125-3-2 487.16

125-6-1 471.80

125-6-2 499.64

0-1.5-1 462.44

0-1.5-2 511.72

0-3-1 473.80

0-3-2 499.36

0-6-1 462.88

0-6-2 483.20

ER-H1

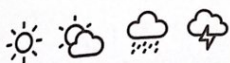
Dot blot 500 ng similar

double compare to 1 min

125-1.5-1 125-3-1 125-6-1 125-1.5-2 125-3-2 125-6-2

0-1.5-1 0-3-1 0-6-1 0-1.5-2 0-3-2 0-6-2





Date / /

Agarose Gel 50mg 120V, 30 min.

(1) 125-1.5-100, 125-3-1, 125-6-1, 0-1.5-1, 0-03-1, 0-6-1

(2) --- -2 -2 -2 -2 -2 -2

28/5 Cell Passage

ER-5 P1+8

(~1 day) 1:4 6-cm dish

1:10 6-cm dish

(~4 days) 1:10 two confocal dish (PDL 5h)

(~1 x day) 1:4 6-well plate (PDL 5h)

29/5 ADPA bleaching

160ul 5uM FAP-5uM MG (45h incubation), 675 nm, 330 W/m<sup>2</sup>, 0, 2, 4, 6, 8 min

PL, PE, UV-Vis of (5uM FAP-5uM MG) x  $\frac{12}{10}$

30/5 Cell labeling

ER-H1 ER P1+8 6-well plate, HI 125 nM, 0.25/0.5/1 mM PA, light 1 min

PA 1 0.25 0.5

1 0.25 0.5

1 ml RNA isoplas, 70 ul MFH20

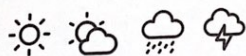
conc. HI 0.25 0.5 0.1

conc. 454.20 496.64 388.20

DMSO 0.25 0.5 1

conc. 436.00 446.32 400.48





Date / /

CuAAC ER-H12, 10 min, 25°C, 500 rpm, column purification (new)

ER-H12	MgH1	0.25	0.5	1
	conc.	424.12	426.92	427.20
	DMSO	0.25	0.5	1
	conc.	423.16	424.16	426.44

31/5 Dot blot (500 ng)

ER-H12 test 27/5 RNA sample <sup>conc.</sup> again

MgH1	1.5	3	6
	391.04	455.20	467.31
DMSO	1.5	3	6
	456.88	405.68	455.20

MgH1	0.25	0.5	1	1.5	3	6
DMSO	0.25	0.5	1	1.5	3	6

Agarose Gel (500 ng), 120V, 30 min

0.25	0.5	1	0.25	0.5	1	1 kb (m)
MgH1			DMSO			

live cell imaging after PA labeling

ER-H12





Date / /

30/5

Cell Passage

NLS-9 P12 1:10 10-cm dish

1:10 6-cm dish

ER-5 P19 1:8 96-well plates X3 for MTT (PDL 6h)

1:4 6-cm dishes X2 (PDL 6h)

30/5

1/6

MTT Assay

MTT-

~60% confluency, MGCL2 20 min

ER-CL2

125, 250, 500, 1000, 2000, 0, empty

3/6 24h later, replace with 90ul colorless media + 10ul MTT

3.5h later replace with 100ul DMSO, 500/550 nm OD

2/6

ER-5 P19 Cell Labeling

6-cm dishes: 125 nM MGHI or 0 nM MGHI

ER-HI

(PA conc.)

1 mM PA; light 1 min.

RNA ex 1 ml RNA isoplas, 100 ul MFH20

125-1-1 0-1-1

1000c. 537.12 507.20

4 tubes: 26.8 ul, 10 ug

Total RNA Isolation (5 tubes of cell lysate in 1 ml RNA isoplas, MS-9), 60 ul

Total RNA 1 2 3 4 5

combine (2)

combine (3)

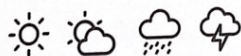
1991.0

1901.5

1664.8

&gt;15 ug/75 ul





Date / /

ER-5 P1+9 6-well plate labeling

ER-H1 MG H1 0.75% 0.5% 1%  
(125nm)  
(PA conc) DMSO 0.75% 0.5% 1% , light 1 min

1ml RNA isoplas, 60ul MF-H<sub>2</sub>O

Cell Passage

MS-9 P1+4 1:20 in 6-cm dish

MS-9 (P1+4) sorted today

① high cell number in 10-cm dish

② low cell number in 10-cm dish

4/6

CuAAC of ER-H125°C, 10 min / <sup>15</sup>min / 20 min / 25 min, 500 rpm, 100ng

ER-H1

new column purification

(CuAAC Time)

conc. 10 min { MG H1 CP } 312.8

{ DMSO (N) } 344.28

15 min { P } 348.20

{ N } 312.04

20 min { P } 337.00

{ N } 345.64

25 min { P } 364.28

{ N } 335.16

Dot blot 500ng

result: 10-25 min similar intensity

→ <sup>15</sup>min - 25 min slightly higher background than 10 min

Agarose Gel: 500ng, 120V, 30min

TAE PAGE Gel: 500ng, 120V, 70 min: similar integrity.





Date / /

## 8/6 Cell Passage

MS-9 new sorte (high) 1:20 6-cm dish

1:3 (500  $\mu$ l) confocal dish.

MS-9 new sorte (low) 1:10 6-cm dish

1:3 (500  $\mu$ l) two confocal dishes1:6 (250  $\mu$ l) two confocal dishesCuAAC (ER-5 H1 175 nm)

ER-H1 PA 0.25, 0.5, 1, 1.5, 3, 6 mM

PA conc. 10  $\mu$ g, 25°C, 500 rpm, 10 min, @ reusol column purification, 20  $\mu$ l NF H<sub>2</sub>O

PA (mM) 0.25 0.5 1 1.5 3 6

conc. 467.52 453.92 467.08 451.60 458.28 378.12

Dot blot 500 ng

Agarose Gel 100 ng, 120V, 30 min

Cell labeling

ER-H1 ER P1+11 0 mM MG H1, PA 0.25, 0.5, 1, 1.5, 3, 6 mM

PA conc. 1 ml RNA isoprep

9/6 RNA extraction, 50  $\mu$ l NF H<sub>2</sub>O

0 mM MG H1 PA (mM) 0.25 0.5 1 1.5 3 6

conc. 485.48 502.40 499.84 503.20 489.60 467.24

CuAAC (ER-5 H1 0 mM)10  $\mu$ g, 25°C, 500 rpm, 10 min, new column purification, 20  $\mu$ l NF H<sub>2</sub>O

0 mM MG H1 PA (mM) 0.25 0.5 1 1.5 3 6

conc. 433.64





Date / /

Dot blot (500 ng)

PA (mM) 0.25 0.5 1 1.5 3 6

MGH1 125 nM

MGH1 0 nM

Agarose Gel (500 ng)

MGH1 0 nM PA 0.25 0.5 1 1.5 3 6

Live cell imaging = MGHBr 500 nM, NLS-9 new sorting (low) 0-30 min. (and high)

10/6 Cell labeling ER Pi+12

(signal outside nucleus)

125 nM MGH1, PA 0.25, 0.5, 1, 1.5, 3, 6 mM.

1 ml RNA isoprep, 60 µl MFH<sub>2</sub>O

0.25 0.5 1 1.5 3 6

conc 658.72 673.24 662.48 625.48 636.48 633.24

Live cell imaging

NLS-9 new sorting (low), MGH1 500 nM, 0-30 min

Cell Passage

new stock NLS-9 Pi 6-cm dish

11/6 CwAAC (ER MGH1 125 nM)

10 µg ~~500 ng~~, 10 min, 25°C, 500 rpm, 20 µl MFH<sub>2</sub>O

0.25 0.5 1 1.5 3 6

conc. 451.28 494.28 503.44 483.22 503.36 534.12

Cell Passage Dot blot: 500 ng

Agarose Gel: 500 ng





Date / /

20/6

MTT Assay

ER-5 P116 (~70% confluency)

96-well plates

ERH1, hv

① No MG, ② MGHI 125 nM, ③ MGHI 125 nM + 1 mM PA

light 1 min, wash twice with DPBS. replace with media

plate 1: 4h later, 981ul colorless media + 9ul MTT, 3.5h

plate 2: 24h later, - - -

Cell labeling

ER-5 P115 ~80% confluency 10-cm dishes

ER-H1

 $\begin{cases} \text{MGHI 125 nM (3ml)} \\ \text{DMSO (3ml)} \end{cases} \quad 20 \text{ min.}$ 

PA 1 mM (newly prepared), 660 nm light 1 min.

① 3 ml RNA isoplus, divided to 3 tubes, resuspend in 60 ul RFLuo

MGHI (125-1-1)

DMSO (0-1-1)

① 1097.4

① 1148.8

② 1046.2

② 1216.4

③ 967.92

③ 1142.7

Mix ①, ②, ③ and re-divided to 3 tubes. Save in -80°C

5/2.5uM 20 min - 10 - 10 min

5uM 20 min - 30 - 10 min

2.5/5uM 15 min - 10 - 10 min

Cell imaging - labeling

hoe-Eq3-DPF in 5% DMSO, 1 ml HBSS (2.5 uM or 5 uM or 0 uM)

incubate 20 min or 15 min, rinse with 1 ml HBSS

incubate 1 ml media, twice (30 + 10 min or 10 + 10 min)

2 mM PA, Green light 1 min (Note: (5/2.5 uM 15 - 10 - 10 min not enough light exposure)) signal is lower than the other three

~~Go to~~ PFA 15 min, 0.1% Triton 5 min, wash twice, blocking 1h, wash twice, ~~Go to~~ 1h, wash (0.1% Triton) X3, DPBS X1, hoechst 3 min, DPBS X1

Result: negative show TAMRA background (spots), all show good localization





Date / /

21/6

DNase + PK

ER-H1

Yesterday isolated RNA (ER-H1)

③ 175-1-1, 59  $\mu$ l, ~1000 ng/ $\mu$ l      ③ 0-1-1, 59  $\mu$ l, ~1100 ng/ $\mu$ l

Add 41  $\mu$ l NFH<sub>2</sub>O, 11.5  $\mu$ l 10X DNase<sup>buffer</sup> (Turbo), 3  $\mu$ l Turbo DNase, 37°C,  
PK 2  $\mu$ l, 42°C, 15 min, 300 rpm  
300-rpm, 15 min. New column purification, eluted with 40 + 63  $\mu$ l NFH<sub>2</sub>O  
(some white solid (column matrix))

③ 175-1-1 - DNase

③ 0-1-1 - DNase

conc. 455.84

555.80

ER-H1

CuAAC

(20  $\mu$ g in 100  $\mu$ l Reaction) X2, 25°C, 500 rpm, 10 min

column purification (reuse the DNase-PK column), eluted with 40 + 62  $\mu$ l NFH<sub>2</sub>O

③ 175-1-1 CuAAC

③ 0-1-1 CuAAC

conc. 410.32

372.28

Set 3 tubes of 10  $\mu$ g RNA in 50  $\mu$ l NFH<sub>2</sub>O aside (-80°C)

Dot blot (500 ng)

Agarose Gel (500 ng)

- CuAAC	MGH1	DMSO
+ CuAAC	MGH1	DMSO

MGH1	DMSO	MGH1	DMSO	1 kb
- CuAAC		+ CuAAC		

22/6

Cell Passage

HEK 293T (from Jinhua) P18 1:6 in three 96-well plates (PDL overnight)

ER-5 P17 1:20 in 6-cm dish

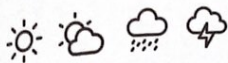
(~100%) 1:8 in 96-well plate (PDL overnight)

MS-9 P15 1:30 in 6-cm dish  
(100%)

remain  
pellet in -80°C

MS-9 P16 (new sort, high) 1:30 in 6-cm dish  
(100%)





Date / /

## Enrichment

Yesterday 7- MGHI / ER 10ug RNA, CI beads 20ul, 1.5h at 25°C  
 ER-H1 (Mix Mode, 18 rate)

Eluted with 10ul MFH<sub>2</sub>O (recover ~ 8.5ul)

Dilute RNA before enrichment to ~  $\frac{100}{200}$  ng/ml (2ug in 20ul)

Qubit 4 CHS)

	+ MGHI(In)	- MGHI(In)	+ MGHI(en)	- MGHI(en)	S <sub>1</sub>	S <sub>2</sub>
conc (1ul)	118	116	too low	too low	51.80	800.63
(2ul)			too low	too low		(500 ng/ml)

(Broad)

	too high	too high	17.2	too low	too low	49.87	766.64
			2ul $\frac{1.758 \text{ ng}}{200 \text{ ul}}$ (0.88 ng/ml)				$\frac{100 \text{ ng}}{200 \text{ ul}}$

$$\frac{0.88 \times 8.5 \text{ ul}}{10 \times 10^3 \text{ ng}} \times 100\% = 7.48 \times 10^{-2}\% \text{ (too low recovery rate!)}$$

## Flow through

Add 200ul MFH<sub>2</sub>O, 600ul Binding <sup>buffer</sup> for column purification (20ul MFH<sub>2</sub>O)  
 + MGHI(FT) - MGHI(FT)

conc.	398.24	418.40
-------	--------	--------

## Dot blot

500ng for input and flow-through, 0.5ul for enriched RNA

input	+	(-)
enrich	+	-
flow-through	(+)	-

Similar level





Date

/ /

23/6

MTT Assay (Dark)

HEK-MG

HEK 293T 178 (0-2000 nM MGHBV, MGHI8, 20 min), replace with  
 24h later (10:30-11:10), replace with 90+10ul MTT, 3.5h.

recycled  
 ↑  
 media

MTT Assay (light)

DBF-ER

ER-5 P1+17

0um DBF, 2.5um DBF, 2.5um DBF + 2mM PA, light 1 min.

DBF incubation: remove media, 100ul HBSS rinse, 40ul DBF/HBSS 15 min.

then 100ul HBSS rinse, wash twice with media 100ul, 10 min each time.

replace with media <sup>→ recycled</sup> (a lot of cells are washed away)

24h later (12:00), replace with 90+10ul MTT, 3.5h.

Enrichment

ER-H1

2x4 = 84ul Cl beads +/- ER-H1 10ug RNA, 20ul beads, 1.5hat20c

A +/- MG-H1 DPBS wash, 60c 5min + 90c 5min (50ul) => 50ul (intotal)

B +/- MG-H1 60c 5min (50ul), (new 20ul) 90c 5min => 70ul (intotal)

(2bit 4 (HS) (1ul)

	A+	A-	B+	B-	S1	S2
(HS) conc.	too low	too low	too low	too low	52.58	829.23
(Bract)	25.8	too low	14.8	too low	51.61	828.94
real conc.	$\frac{3.11}{200ul}$		$\frac{1.785}{200ug}$			$\frac{100ug}{200ul}$

$$\frac{3.11 ng/ml \times 8.5ul}{10 \times 10^3 ng} = 0.26\%$$

$$\frac{1.785 \times 8.5}{10 \times 10^3 ng} = 0.15\%$$

yesterday 0.075% @

Flow through: 150ul NF120, column purification, eluted with 20ul NF120

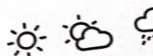
con. A+ A- B+ D-

518.84

111

B+

D-



24/6

Cl





① in 160  $\mu$ l NF H<sub>2</sub>O 1034.1 ng/ $\mu$ l

② in 70  $\mu$ l NF H<sub>2</sub>O 754.4 ng/ $\mu$ l

Set 40  $\mu$ g in 80  $\mu$ l NF H<sub>2</sub>O aside

### Cell labeling (Protein MS) HLB

MS-9 R14 two 15-cm dishes ( $\sim$ 90% confluency)

4ml 125 mM MGHI, 2 mM PA or 0 mM PA, 66  $\mu$ M 1 min  
(+6  $\mu$ l 10 $\times$  PK)

half: 600  $\mu$ l 1% SDS HEPSE buffer, sonicate

half: 300  $\mu$ l HLB (+30  $\mu$ l 10 $\times$  PK), on ice 10 min (rotate),  
supernatant + cyto

800 G 8 min, then wash twice with HLB (500  $\mu$ l), 200 G,

2 min. to the pellet add 300  $\mu$ l 1% SDS HEPSE (+30  $\mu$ l

10 $\times$  PK), sonicate.

17/12 Test conc. with BCA kit

Total +/-

Cytosol +/-

Nucleus +/-

1.5962/1.8101

3.7474/3.6325

1.1512/1.2951

### CuAAC

80  $\mu$ g in 150  $\mu$ l, 37 $^{\circ}$ C, 500 rpm, 1 h. add 37.8  $\mu$ l 5 $\times$  loading buffer

95 $^{\circ}$ C, 300 rpm, 5 min denature, fast cool down on ice

10

### 10% SDS - Page

one 20  $\mu$ l protein, 2  $\mu$ l marker for blue staining

one 20  $\mu$ l protein, 6  $\mu$ l marker for western blot (biotin)

80 V 40 min, 130 V 70 min.

transfer: 20 V, 1 A, 45 min

milk 45 min, anti-biotin 1 h, anti-rabbit 1 h.

biotin  $\sim$ 20 min: signal in nucleus and cytosol.





Date / /

19/12

Cell labeling C Protein MS) HLB

MS9 P15 one 10 cm dish (~90% confluency)

with HLB containing 0.3% tween-20, but not 0.3% NP-20.

other parameters keep in the same

125nM MG132, 3min PA, 660-~~nm~~ 1min. wash twice with PBS

{ half: 600 ul 1% SDS HEPES, 60 ul 10xPK, sonicate

{ half: 300 ul <sup>(tween-20)</sup> HLB, 30 ul 10xPK, 10min on ice, 1000G, 5min

the supernatant as "cyto", remain wash twice with

<sup>(tween-20)</sup> HLB, 200G, 4 min. Then add 200ul 1% SDS HEPES,

20 ul 10xPK, 10 sonicate.

Cell labeling MS protein MEM

MS-9 P15 one 10-cm dish (~90% confluency)

with Thermo ~~membrane~~ Mem-Port<sup>TM</sup> Plus Kit

{ half: 600 ul 1% SDS HEPES, 60ul 10xPK, sonicate

{ half: 300ul permeabilization buffer, 30 ul 10xPK, 10 min on ice

16,000XG 15min. The supernatant as "cytoplasm".

Remain 10 add 200ul solubilization buffer, 20 ul 10xPK,

pipetting, <sup>on ice</sup> 400 for 30 min, 16,000G for 15 min, supernatant

as "membrane". Remain add 150ul 1% SDS HEPES, 150ul

10xPK, sonicate.

## BCA Assay

HLB (Total Cytosol Nucleus) MEM (Total Cytoplasm membrane Nucleus)

1.1215 0.5548 0.9594

1.1942 0.7820 0.7858 1.2938

600 CuAAC: (biotin-azide in DMSO)

62ug in 150ul, 500rpm, 37°C, 1h.

Then add 37.5ul 5X loading buffer, 95°C 5min, 300rpm denature.





Date

/ /

RT: 500 ng RNA, dilute cDNA with 30  $\mu$ l MF H<sub>2</sub>O

22/12 qPCR: 2.5  $\mu$ l cDNA

primer: XIST, ~~MEAT~~<sup>CAPI</sup>, GAPDH, ACTB

not stable

Cell labeling: HLB (tween~~20~~ or NP40) MS-9 P+5

125 nm MG-H1, 3 mM PA, 1 min light

Fractionation:

200  $\mu$ l HLB (with 0.1%, 0.05% Tween-20 or NP-40), rotate on Tle for 5 or 12 min. ~~800G~~ 1,500G (8000 rpm) 4 min. Save the supernatant as cytosol (add 10x PK before store in -80°C)

Wash the pellet with 500  $\mu$ l HLB buffer, ~~800G~~ incubate on Tle for 5 min, 800G 3 min.

Add 200  $\mu$ l 1% SDS HEPSE, sonicate, as nucleus.

Cell Passage

MS9 P+5 1:5 in ~~two~~<sup>one</sup> 10-cm dishes

23/12 test the conc. by BCA Assay (37°C, 1h)

NP-40	0.1% (5min)	MS	<del>cyto</del>
		0.8678	1.5176
	0.05% (5min)	0.6488	0.9239
	0.05% (10min)	0.6685	0.9674
Tween20	0.1% (5min)	1.0095	0.3148
	0.05% (5min)	1.0713	0.3372
	0.05% (10min)	1.1134	0.4074



CNA

73

25/12 Ce

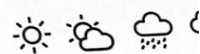
27/12 S

to

L

28/12





then 500G 5min. Save the supernatant as cytoplasm.

500ul MEM + 5ul PK, vortex 5s, incubate on ice for 5min, 3000G for 5min. Save the supernatant as membrane

250ul MEM + 2.5ul PK, vortex 15s, incubate on ice for 30min. <sup>150ul</sup> Save  
5000G 5min. the remain add 1% SDS HEPSE, sonicate.

BCA Assay 37°C. 45min

Total Cyto MEM soluble NLS chromatation

1.9855 1.0380 0.8880 0.8404 1.5384 ~~1.5384~~ ug/wl

Cell labeling CMS - Protein MEM kit

10-cm dish.

half 400ul MEM (5ul PK), on ice 5min. 16,000G, 5min

half 400ul MEM (5ul PK), on ice 10min. <sup>16,000</sup> ~~5000~~G, 5min

then 400ul MEM soluble solution (5ul PK), on ice rotate 50min.

16,000G 5min. ~~Save~~ remain 400ul 1% SDS HEPSE sonicate (5ul PK)

10min

5min

Cyto 1.0068 0.9248

MEM 0.8637 0.8657

NLS 1.1619 1.0859

2/12 CuAAC with N<sub>3</sub>-545

(10.1)

0.4mM 545-N<sub>3</sub> instead of Biotin-N<sub>3</sub>.

Subcellular kit & extracted protein. (75ul reaction, <sup>56.7</sup> ~~46.7~~ ug protein)

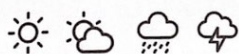
37°C, 500rpm. 1h.

CuAAC (2/11 total ER +/- 1.6261/1.6499

0.1mM @ 545-Azide, <sup>37°C</sup> ~~67°C~~ 25°C, 500rpm 1h

50ul reaction





Date / /

## 50ul Reaction (high)

CuSO <sub>4</sub> (120mM)	to 1mM	0.42ul	} 7.25ul
THPTA (46mM)	to 2mM	2.17ul	
545-Azide (10mM)	to 0.1mM	0.5ul	
NaAsc (200mg/ml)	to 16.7mM	4.17ul	

## 50ul Reaction (low)

CuSO <sub>4</sub> (20mM)	to 333uM	0.884ul	} 3.32ul
THPTA (46mM)	to 666uM	0.724ul	
NaAsc (100mg/ml)	to 2.5mM	1.25ul	
545-Azide (10mM)	to 100uM	0.5ul	
or Cy5-Azide (1mM)	to 100uM	5ul	7.82ul
Protein used: ~64ug, 40ul			

or only 40ul protein with 10ul NF H<sub>2</sub>O

Reaction at 30-25°C, 500 rpm for 1h. then add 12.5ul 5X loading  
~~denature~~ without denature

## 8% SDS-Page

load 16ul (+4ul 5X loading dye) protein without denature to gel. 2ul Marker  
~~120V~~ 80V 40min, 120V <sup>75</sup>~~40~~ min.

\* 545-Azide move slightly faster than loading dye

left to right:

① 1Kb, total, cyto, membrane, nucleus soluble, chromatin

②	ER+	ER- (no CuAAC)	ER + / -	ER + / -	ER + / -	1Kb
	empty	Cy5, 545	high (545)	low (545)	low (Cy5)	

③ wash with PBS, 3 times, 5 min.

Result: ① signal every fraction

② Cy5 show higher background

Cy5 Marker / <sup>loading dye</sup> interference Cy5

Marker 75KD interference 545





30/12 CrAAc. Then 12% SDS Page  
 CrAAc: 25°C, 500 rpm, 1h. Then in R.T. 0 rpm (~30 min)

ER-(2) 31.4 ug 50ul

total +/- total +/- Cyto +/- membrane +/- others +/-  
 no Cu

NLS 40ug 50ul (MEM kit) all positive

total total Cyto mem NLS Cyto mem NLS 1kb  
 no Cu 10 min 5 min 2ul

NLS 30ug 50ul (HLB) all positive

~~total~~ 1kb total total 0.1% Cyto 0.1% NLS 0.05% Cyto 0.05% NLS 0.05% Cyto 0.05% NLS  
 1ul No Cu AP 40 5 min AP 40 10 min AP 40 5 min

PK 0.1% Cyto 0.1% NLS 0.05% Cyto 0.05% NLS 0.05% Cyto 0.05% NLS  
 0.5ul tween-20 5 min tween-20 10 min tween-20 5 min

All 16ul protein + 4ul 5X loading dye

except tween-20 Cyto 32ul protein (only 15ul in 50ul)

SDS-Page: 80V 1h, 120V ~90 min

Cell labeling (NLS protein) HLB (0.05% tween), MEM kit

10-cm 250 nM MG-H1, 3 mM PA, 0/2 min hv, wash twice with PBS  
 100% confluency 500ul total.

3 mL PBS 1200 ul HLB (0.05% tween) 1000 rpm 3 min  
 1300 ul MEM kit

wash once with 500ul wash buffer (from MEM kit) 1000 rpm

HLB: Add 300ul HLB, incubate 5 min, 1600 G 5 min.

the supernatant, add 2.5ul PK (from subcellular kit), save as Cyto

Wash pellet with 300ul HLB, incubate 5 min, 800 G 5 min (kit)

the dissolve in 0.5% SDS HEPES 400ul. Sonicate



(ME)

for

as

for

for

(Tot)

Col

3/12 B

tot

+

-

C

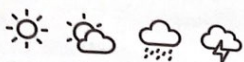
4

7

2

12





Date / /

MEM kit: Add 300  $\mu$ l permeabilization buffer, incubate on ice for 10 min, 16000 G 5 min. Add 2.5  $\mu$ l PK to the supernatant, save as cyto. Then add ~~200~~<sup>200</sup>  $\mu$ l solubilize buffer, incubate on ice for 30 min (with 2  $\mu$ l PK), 16000 G 15 min, save as membrane. remain add 400  $\mu$ l 0.5% SDS HEPES, sonicate. add ~~4~~<sup>5</sup>  $\mu$ l PK

Total: add ~~400~~<sup>500</sup>  $\mu$ l 0.5% SDS HEPES, sonicate. add ~~5~~<sup>5</sup>  $\mu$ l PK

### Cell Passage

MS-9 Pit9 1:10 in three 10-cm dishes.

0.05% MS  
✓  
5 min

### 3V12 BCA Assay (45 min 37°C rpm)

	total	total	HUB cyto	Wash	MS	MEM cyto	mem	MS
+	1.0709		0.4933	1.1524	1.9508	1.7255	1.4492	1.2566
-	0.8659		0.5046	0.9882	1.6926	1.6213	1.3246	1.2589

CuAAC  $\rightarrow$  HUB cyto 22.6  $\mu$ g

40  $\mu$ g protein, add  $\text{H}_2\text{O}$  to 46.2  $\mu$ l, denature in 90°C for 5 min, on ice

Then prepare CuAAC solution (total +/- one set as without  $\text{CuSO}_4$ , THPTA, NTA) 25°C, 500 rpm, 1 h.

12% SDS-Page: (MEM 1 mm, HUB 1.5 mm)

16  $\mu$ l protein + 5  $\mu$ l (5X loading dye)  $\rightarrow$  20  $\mu$ l to wells.

(HUB cyto 30  $\mu$ l protein + 10  $\mu$ l 5X loading dye  $\rightarrow$  40  $\mu$ l to well.

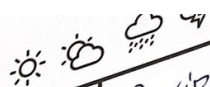
80 V 40 min, 120 V 2 h.

### Cell Passage

ER-5 Pit1 from frozen stock in 6-cm dish (~1:15)

it)





### Precipitation:

110ul W. ② (~200ug) in  
-80°C for 30min, 15000G 10min, 2X  
resuspend in 10ul 4% SDS-HEPES + 200ul DPBS: can res.

### CuAAC MS-MEM

83ug protein in 100ul reaction, 25°C, 500rpm, 1h.

Wash with acetone: (500ul chill acetone, keep in -80°C  
for 30min, 15000G 15min) X2

Some solid still in slight pink.  
resuspend in 10ul 4% SDS-HEPES + 40ul 0.1% SDS-HEPES.  
Store in -20°C

5/1

### Cell Passage

ER-5 Pt3

1:4 in two 10-cm dishes

1:5 in two 10-cm dishes.

one 6-cm dish

### Cell labeling ER MEM kit

ER-5 Pt2 ~95% in two 10-cm dishes

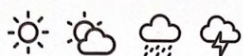
250nm Mg-H2, 3mM PA, 2min or 0min hv

{ 250ul as total (add 300ul 0.5% HEPES sonicate) (10x PK)  
21.8ml MEM kit (10x PK)

Wash once with wash buffer.

Then 800ul cell permeabilization buffer, 10min on ice, 16000G  
15min. Then 400ul solubilization buffer, 30min on ice, 16000G  
15min. Then 400ul 0.5% HEPES, sonicate.





Date / /

## BCA Assay

total +/-	Cyto +/-	mem +/-	others +/-
1.4235/1.1403	1.2139/1.3212	1.2675/1.3836	1.7192/1.8827

save in -80°C

b/1

## ER-MEM

additional mem +/- without wash

83ug protein in 100ul Reaction, 25°C, 500rpm, 1h.

<sup>(92.4ul)</sup>  
Wash with acetone: (500ul chilled acetone, 0.1u/wash in -80°C for 30 min, 15000 G 15 min) x 2

still some solid in pink slightly

resuspend in 10ul 4% SDS-HEPES + 40ul 0% SDS-HEPES.

together with sample prepared yesterday (NLS-MEM), heat in 75°C oven for 5 min. to dissolve

BCA Assay = 30 min 37°C

	NLS	ER
total +/-	1.6482/2.1961	2.3201/1.6803
Cyto +/-	2.7669/2.8620	2.5217/2.3020
MEM +/-	3.0241/2.3087	2.9035/3.0523
Other +/-	2.0756/2.0635	1.9711/2.0809

## 12% SDS-Page

Sample preparation: 20ug protein in 12ul 4% SDS-HEPES, add 12ul 2x loading dye (BioRad), 90°C 5 min denature

For mem +/- (ER) without wash: (12ul (~10ug) + 12ul 2x loading dye) x 2 { one denature at 90°C, 5 min

| one without denature.





ER:-

total +/- Cyts +/- mem +/- other +/- empty 1ul Marker

NLS

total +/- Cyts +/- mem +/- other +/- empty 1ul Marker

ER:

mem +/- (wash) → mem +/- (no wash) → mem +/- (no wash)  
 (denature) (no denature) (denature)

→ empty → ~1 ul Marker → empty → ~3 ul marker

80V 40 min, 120V 2h. Wash 3 times with H<sub>2</sub>O. capture image.

blue staining overnight: no signal.

change to Ag staining: still no signal

7/1 Cell Passage

ER-5 Pt4 1:5 in two 10-cm dishes  
 one 6-cm dish.

Cell labeling ER - MEM kit

ER-5 Pt3 250 nM MG12, 3mM PA, 2min L.V

MEM extraction

(250ul at total (300ul 0.5% HEPES buffer) (10xPK)  
 175ul for MEM extraction (10xPK)

800ul permeabilization buffer, 10min on ice, 16000G 15min.

400ul solubilization buffer, 30min on ice, 16000G 15min.

400ul 0.5% HEPES, sonicate





80V 40min, 120V 2h (can be ~110min next time)

Blue staining: page blue 2h. Wash overnight

Cell labeling ~~MIS~~ - HLB buffer (10xPK)

MIS 9 P+13 ~95% confluency in two 6-cm dishes.

250mM MG-H1, 3mM PA, 0 or 2 min hv

Cell in DPBS, 200G 4 min.

Replace with 250  $\mu$ l 0.5% Tween-20 HLB, rotate on ice for 10min.

1000G 4min, Save as cpts.

Replace with 250  $\mu$ l 0.5% NP-40 HLB, rotate on ice for 10min.

1000G 4min. Save as wash.

Replace with 400  $\mu$ l 1% SDS-HEPES. sonicate

save in - ~~80~~ 20°C.

Cell lysis

MIS-9 P+13 ~95% in one 10-cm dish.

Add 2 ml DPBS, 200G 4min. Replace with ~~0.5~~ 0.5 ml  
0.5% SDS-HEPES / or ~~0.5~~ 0.5% HLB  
Sonicate (1 min 20s) X 2.

MIS-9 P+13 ~100% in one 6-cm dish 50ml

Add 1 ml DPBS, 200G 4min. Replace with 0.5 mL DPBS.  
Sonicate (1 min 20s) X 2, no clear

{ HLB

{ HEPES

{ DPBS





Date / /

## 9/1 Cell Labeling <sup>ER</sup> ~~NLS~~ - HLB and MEM

ER-5 P+4 ~95% in 10-cm dish

250nM MGHI, 3mM PA, 2min hv

{ half MEM kit (10xPK)

{ half HLB (10xPK)

Wash once with MEM Wash buffer, 200G, 4min

MEM:

400ul permeabilization buffer, rotate 5 min on ice, 16000G <sup>10</sup>15min,

Save as cyto. 2400ul solubilization buffer, rotate 30 min on ice, 16000G

15 min. Save as mem. remain add 3400ul 1% SDS-HEPES, sonicate.

HLB:

250ul 0.05% Tween-20 HLB, rotate ~~500~~ 10 min on ice. 1000G 4min

Save as cyto. 250ul 0.05% NP-40 HLB, rotate 10 min on ice, 1000G 4min

save as wash. 400ul 1% SDS-HEPES. Sonicate.

## Cell lysis

~~NLS~~ ER-5 P+4 ~95% in on 10-cm dish { half HLB 0.5ml

{ half 0% SDS-HEPES 0.5ml

ER-5 P+4 ~95% in 6-cm dish: 0.5ml DPBS

sonicate (1min 20s) x2, not clear.

{ HLB

{ HEPES

{ DPBS.

HLB, +/-

## BCA Assay

8/1 NLS: HLB HEPES DPBS Cyto +/- ~~mem~~ Wash +/- nucleus +/-

1.9080 1.8372 1.8319 0.9864/0.8526 1.8136/1.7292 1.6143/1.4883

9/1 ER: HLB HEPES DPBS Cyto+ <sup>HLB</sup> wash+ nucleus+ <sup>MEM</sup> cyto+ mem+ other+

2.0037 1.9381 1.5357 1.4924 1.7926 1.3744 1.2499 1.7008 1.0663





Date / /

Cell Labeling for RNA

500ul 5uM EG3 EG3  $\rightarrow$  no PA L4  
EG1 DMSO

30 min, 2mm PA, 2min hv, 1ml RNA iso Plus.

~~etc~~ in 60ul MF-H<sub>2</sub>O

EG3 EG1 C4 DMSO EG3 no PA

531.24 577.68 512.06 556.12 548.64

## 11/5 Cell Passage

HEK 293T ATCC P1+4  $\checkmark$  1:5 in 6-cm dish

MS-9 / ER-5 P1+2

1:2<sup>20</sup> in 6-cm dish

1:2<sup>20</sup> in two confocal dishes (PDL)

## 12/5 Live Cell Imaging

HEK 293T three confocal dishes

F-AQ 10uM (0.5% DMSO), 1h.

hoechst 8uM

empty (only <sup>DMSO in</sup> HBSS)

13/5 Live cell imaging ER-MG-H2, MS-MG-H2

ER/MS: 500nM MG-H2, 20min colocalize to mcr3

MS: 500nM MG-H2, 20min, then 8uM hoechst

(colocalization of MG-H2 and hoechst)

ER: 500nM ER-Tracker Red in HBSS, 35 min, colocalize to

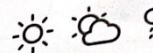
mcr3. Then add 500nM MG-H2, colocalize <sup>Tracker</sup> ~~mcr3~~ to mcr3

Cell Passage: HEK, MS-9, ER5 1:5 6-cm dish

## 15 PA/5 Cell Labeling for imaging 1:4 two @ 6-well plates

coverslip 150ul, well 500ul, 1h 5uM hoe-EG3-DBF,

2mm PA (6 min), hv 2 min or 0 min



coverslip

no coverslip

16/5

15

C

T

S





6-well plate

Date / /

	HEK	MS-9	ER-5	HEK	MS-9	ER-5
cover slip	150ul	150ul	150ul	150ul	150ul	150ul
no cover slip	500ul	500ul	500ul	500ul	500ul	500ul
	+hv			-hv		

{ Cell imaging: Fix with 4% PFA, ---- result: no difference among cell lines.  
Protein @: wash twice with cold DPBS, 2000 rpm, 5 min.

Add 80ul 4% SDS HEPES, save in -80°C

Cell Passage

HEK 293T ATCC R4+12 }  
MS-9 R1+4 } 1:20 in 6-well dish  
ER-5 R1+4 }

16/5 Protein labeling CuAAC

15/5 cell in 80ul 4% SDS HEPES (with ~20ul DPBS), add 200ul 0% SDS HEPES, sonicate 1 min 45s. add 30ul 10x PK inhibitor

Test conc. via BCA Assay

HEK +/- MS +/- ER +/- hv

0.7725/0.8573 0.523/0.49/5 0.7561/0.7775

(also test ER sub total + 7/1 1.8269 : 1.8715 today)

CuAAC (100ul 46ug)

46ug for MS, 70ug for others, 37°C, 1h, 500 rpm.

add 33ul 4x loading buffer, 90°C, 5 min. cool down on ice

SDS - Page (12%) 1.5mm

HOE-31735

① 2ul Marker

ER-MG+1

ER

HEK

MS

30ul

30ul

30ul

46ul ~15ug

② 2ul Marker

ER hv+/-

HEK hv+/-

MS hv+/-

20ul

31ul

~10ug

80V 40min, 100V 2h 20min





Date / /

RNA CnAAC

10/5 label and isolated

10ug, 25°C, 10 min, reused column purification, 20ul MF420

EG3	EG1	C4	DMSO	EG3 no PA	EG3 no hv
516.16	521.56	519.96	515.96	505.36	491.00

Dot blot: 500ng

(1) EG3 EG1 C4 DMSO no <sup>hv</sup> no ~~PA~~

(2) EG3 EG1 C4 DMSO

EG3 no <sup>hv</sup> no ~~PA~~ DMSO

Agarose Gel: 500ng

EG3 EG1 C4 DMSO no hv no PA

17/5 Protein SDS Page, redensatured in 90°C for 3 min.

8/5 MS-H1 + 30ug/625ul = 0.48 ug/ul

16/5 ~~ER~~ ER, HEK + 70ug/133ul = 0.525 ug/ul

Marker	x	x	MS-H1	ER	HEK
2ul			20ul	20ul	20ul

80V 1h, 120V ~ 1.2h.

18/5 Protein CnAAC

MS-MS-H1 (500nm, 3min PA, 2min hv, 2/1 labeled) 0.8558 mg/ml

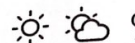
HEK 293T / ER 15/5 labeled conc. 0.7725 / 0.7561 mg/ml

CnAAC: 35ug in 500ul, 500rpm, 25°C, 1h.

17ul 4x loading buffer 95°C 5min

12% SDS-Page: 1ul Marker x x MS HEK ER

80V ~~4h~~ 3h 15 min



19/5

21/5

22/5





Date / /

result: M4H1 in higher signal

19/5

ER HEK M4H1 (MS) x Marker 1u1

hoeDBF

25°C (18/5)

37°C 10 ug / 20ul  
(16/5)

0.12% SDS-PAGE: 80V

## Cell Passage

MS9 / ER-5 / HEK 293T 1:20 in 6-cm dish

P4+5 P4+5 P4+13 1:10 in 6-well plate (2 wells PD22x)

1:5 in 6-well plate (PDL2x, coverslips)

21/5 Cell labeling hoeDBF wash or not

5uM hoe-EB3-DBF

✓

✓

✓

0.5% DMSO

✓

✓

✓

30min incubation

15min incubation

30min incubation

15+15 min wash

15 min wash

wash once with HBSS, 2min PA, 2min. wash twice

fix 15 min, 0.1% Triton 10+5 min. DPBS 5+5 min. blocking 30min

DPBS 5+5 min. AntiAc 1h. @ 0.1% Triton 5min x 3. hoechst 1/1000

3min. DPBS 5+5 min

result: wash can reduce signal in cytosol

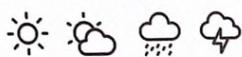
22/5

## Cell Passage

MS9 P4+6 ER P4+6 HEK 293T P4+14 1:20 in 6-cm dish

1:8 in 6-well plate (2 wells PDL0, coverslip)





Date / /

## Cell labeling

HEK ~~MS~~ ER MS 1st

2nd

5uM Hoechst DAPI 30 min, 20 min wash x 2

2mM PA, 2min hv. @ 3000 rpm wash x 2. (combine MS 1st and 2nd)

4% SDS HEPSE 50ul, store in -80°C

because of low %

23/5

## Protein CNAAC

@ 23/5 @ cells, add 150ul 0% SDS HEPSE, sonicate 1 min 45s.  
add 20ul 10x PK inhibitor. BCA Assay

ER 1 2 HEK 1 2 MS 1-2

0.7495 0.8117 1.0917 1.0262 0.7261

4% MS-MG-H2 (0 min or 2 min hv) +/- 0.9061 / 0.9967

2% MS-MG-H2 <sup>2min hv</sup> + 0.8558

30ug in 50ul reaction, 37°C, 500 rpm, 1h.

for MG-H2 (MS): can also test 37°C, 500 rpm, 20 min + 4°C 40 min

Add 16.7ul 4x loading, 95°C denature 5 min

## 12% SDS - Page

1ul Marker x MG-H2 0.1h, 20min HEK ER ER HEK MS

60V 50min, 80V 100V 1h, 120V 40min

result: 20min and 1h MG-H2 in the same signal  
loading control HEK > MG-H2 > ER and MS

use blue stain solution from XCLi group, works well. (microvane 2min  
+ 10min incubate on shaker)

## Cell Passage

HEK-Pi





Date / /

24/5 12% SDS-Page

①	Marker	x	MG-H1 (MS)	HEK	ER	MS
	1ul		<del>20ul</del> 18ul	<del>18ul</del> 24ul	<del>20ul</del> 22ul	<del>20ul</del> 22ul

②	Marker	x	MG-H1 (MS)	HEK2	ER-2	MS
			18ul	18ul	24ul	22ul

60V 1h, 100V 1h, 120V 40-45min

25/5 Labeling for imaging MG-H1 (MS and ER)

125nM MG-H1 in 1ml DMEM, 20min. Rinse with HBSS,

2x 15mm PA, hv 1min.

MG-H1	MG-H1	DMSO
	→ no PA	
MG-H1	MG-H1	MG-H1 (HBSS)
	→ no hv	
	no hoechst	

50V 1h, 100V 1h, 120V 40-45min

Labeling for imaging HoeDBF (HEK 293T)~~500nM~~ 5nM Probe in 200ul HBSS (0.05% DMSO), 30min

rinse once, 20min media x 2. Rinse once. 2mm PA, hv 2min

fix 3 6-well plates with 4% PFA for 15min. 0.1% Triton 10min + 5min.

Wash 5min x 2. Blocking 30min. Wash 5min x 2. OVAAC 37°C 1h.

0.1% Triton 5min x 3. hoechst 1/1000 3min. Wash 5min + 10min.

Cell Passage

HEK 293T P4 + 145, ER-5 / MS 9 P+7 1-5 6-cm dish.