

Smithies

OLIVER SMITHIES

DEPT. OF PATHOLOGY

CB# 7525 UNCAT CHAPEL HILL

NC 27599-7525

June 15th, 1999.

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Made in U.S.A.

Oligo # 22842 5' Oligo Name: J'1 Blue 3'
 Date made: _____
 LENGTH: 19
 % G-C: 42%

T	G	A	T	G	C	T	T	C	T	A	T	G	C	C
A	A	G	A											

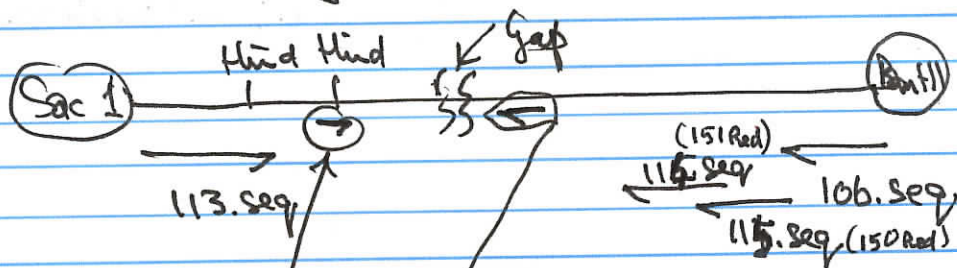
Oligo # 22843 5' Oligo Name: J'1 Red 3'
 Date made: _____
 LENGTH: 20
 % G-C: 40%

G	G	T	G	C	T	T	C	A	A	G	A	A	C	A
T	T	G	T	T										

Page

Wed ~~Thurs~~. June 16th

Extending sequence of CRLRMUS



Need new primer from 3' end of 113. seq.
 " " " " 5' end of 115. seq.

M.P. $56 + 22 = 58$ J1 Blue across the hind CRLRMUS. SEQ 6552 to 6570
 M.P. $28 + 24 = 56$ J1 Red from 7264 to 7245
 See if mp. has any significant effect

Thurs. June 17th Nancy Elizabeth's day!

Submitted noon	J1 Blue	Seq 116	14 μ l water	1 μ l I'143 27.3	5 μ l J1 Blue 100

Submitted noon	J1 Red	Seq 117	14 μ l Water	1 μ l I'143 27.3	5 μ l J1 Red 100
				Template Primer	

A handwritten diagram on a piece of paper. At the top, the text "mixed classes?" is written. Two arrows point downwards from this text to the words "Expected" and "3.3k". To the right of "Expected" is the text "Expected 4.4". A bracket on the right side of the diagram groups the "Expected" and "3.3k" labels, with an arrow pointing to the text "Expected out of 1.7" written above the bracket.

$$\frac{RV}{C_{\text{air}}} \left\{ \begin{array}{l} \\ \\ 27.5 \end{array} \right\}$$

① Conclusion: looks as if the I³ Pool was a mistake - there is something else in there in addition to expected. However expected is OK, so proceed. See I¹ 57.
RV/CLA (Pr) is shorter than 27.5 Å

(2) Con

8x8

878

① 21:30
~~22:30~~
~~23:30~~
Duo
W
Agm
W
J3
J3
J3
27.5 I1
I1
W
Agm
W
B

Nancy
Thurs
Picked (12) - 6
to 18A
R.T. 9
329
Pro

37° Tues June 22nd 2:40 PM to —

$k+31$

More work on thing to cla

Take 20% for Eco RI cuts

✓ At Spr I'bi Pool

+ 1 pl Hind III lot 51 (200/ pl)

32nd 10:25^{am} to 11:20^{am}

$\begin{matrix} \text{I}^3 & \text{C} \\ \text{P Hund} & \text{A} \end{matrix}$

1:20 pm

$$\frac{\phi_{OH}}{H_2O} = 2 \times 10^{-4}$$

CIAA

Note: I'60 Hind (27.5) is at same stage as; it was made from (27.5) mini after post/HQ, CIAA.

J^+3 (Blue) $\rightarrow$ I 160 (Red) 27.5 (OH/HOQ 2x CIAA)

Take $\sqrt{1 \text{ pl } 10 \times p95 \text{ hq. buffer}}$ $\sqrt{1 \text{ pl}}$
+ $\sqrt{8 \text{ pl dhd}}$ $\sqrt{8 \text{ pl}}$

High

✓ 4 ✓ 1 pe T 3 P Hybrid
✓ 1 pe T 156 blind → 1 pe T 16
✓ 1 pe T 4 H gate ditale lot 970 1 pe ✓

CIAM
in 27.5

15° 1:55 pm to 3:45 pm, R.T. 3:45 pm to 4:50 pm, 37° 4:50 pm to 5:40 pm (27.5) hig.

27.5 h_{eq}

Transformer

I 139 FHB 446

Blue

~~Red~~ Black



Nancy's day
Thurs. June 17th

Picked (1)-(16) Blue (P)
(17)-(24) Black (27.5)

to 18 Amp N ZY

R.T. 9:40 to 1:45 pm

320:45 9a

Kate ~~Blue~~

$\frac{1}{2} \times 20 = 10$

(Black) ⁽¹³⁾ R, ⁽²⁾ 20, 5, 20/16, 5/16

NZY 1xtra

22nd 6:30h

11/15/20

Proceded to 50 pl & to cold. 1x POH/HOQ, 2x CIAA (strong) $\uparrow$ 16

$$\frac{1}{k+24} \text{ mR } (1 \text{ on } 20)$$

10 am $\leftarrow$ R.T. until 2:50 pm 4.25 hr $\leftarrow$ left open for 6 hrs

9 am 10:5237
V. wet
Hales
IN 241 (Hales)

4

J3

①

②

Blue

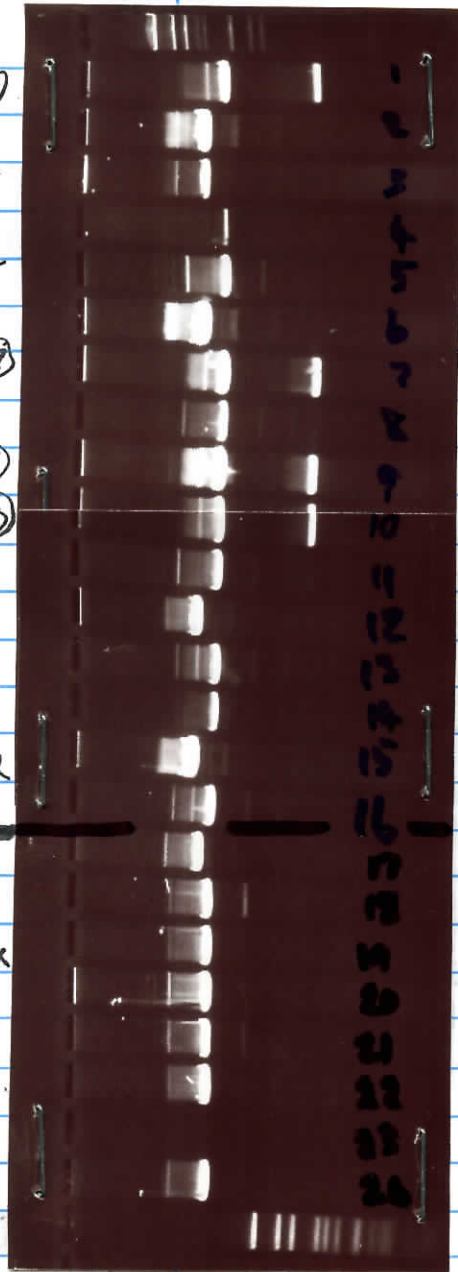
③

④

⑤

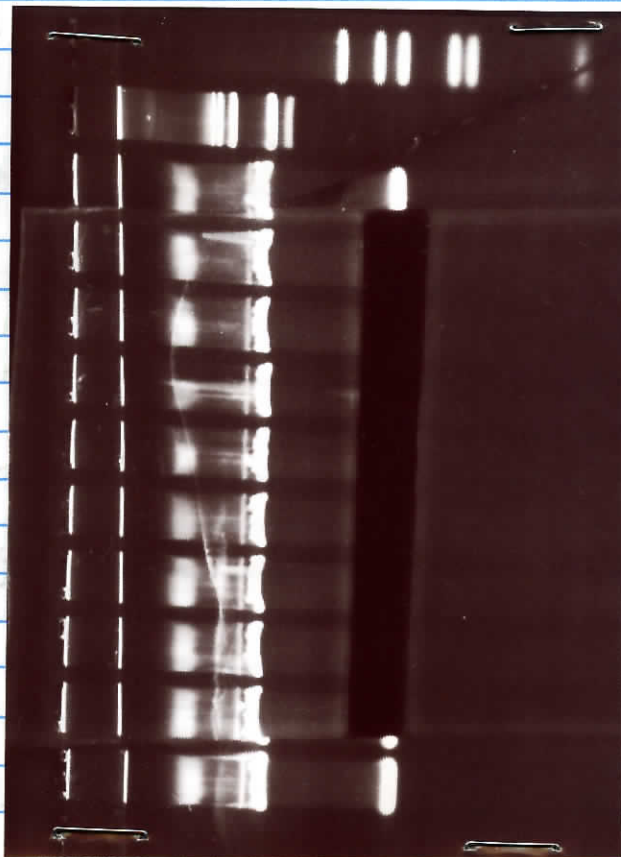
Blue

Black



①

Conclusion: The control will need to be repeated, but ①, ⑦, ⑨ & ⑩ Blue are good. Reformat the Black, control, hi.g. & try again. Meanwhile make more of ⑨ Blue overrule.



②

Conclusion: H: 23 was no good either. Good 1.1 kb. Cla from ⑨ & excision.

①

②

③

④

⑤

⑥

⑦

⑧

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① ~~no~~ ~~off~~ ~~1.2~~ ~~max~~ ~~RNAse~~ 3/10
 1.2 ~~max~~ ~~RNAse~~ 3/10

Sun - June 20th

Test on 5'3 minis

Take 50 μ l 10x ~~NEB~~ NEB Buffer 4
 50 μ l 10x Aclb
 390 μ l ddw

10 μ l (50 μ l) Cla I lot 33 (50 μ l) (20/Rx)

20 μ l / mini 1 μ l J'3 37° 4:40 to 5:40 then back to 37°. Cld 7:30

① - ①⑥ Blue ①⑦ - ②④ Black - Expect single Cla on failures.
 1.1 kb 2 (smaller) Cla on Blue & 2 (larger) Cla on Black (1.6 kb).

If successful set up overnite for prep. gels

Take 20 μ l 10x NEB buffer 4
 20 μ l 10x BSA
 156 μ l ddw
 4 μ l (20 μ l) Cla I lot 33 (50 μ l)
 + 10 μ l mini J'3 #⑨ Blue

J'5
#⑨
Cla

& re-set up mini #23 with excess of dd Cla mix.
 37° 7:30 pm - Jan Ma

Mon - June 21st

2/10:25 am
 off 1:15 am
 5V on

② Dye
 W
 HCR MM
 J'5 23
 J'5 #⑨ Cla

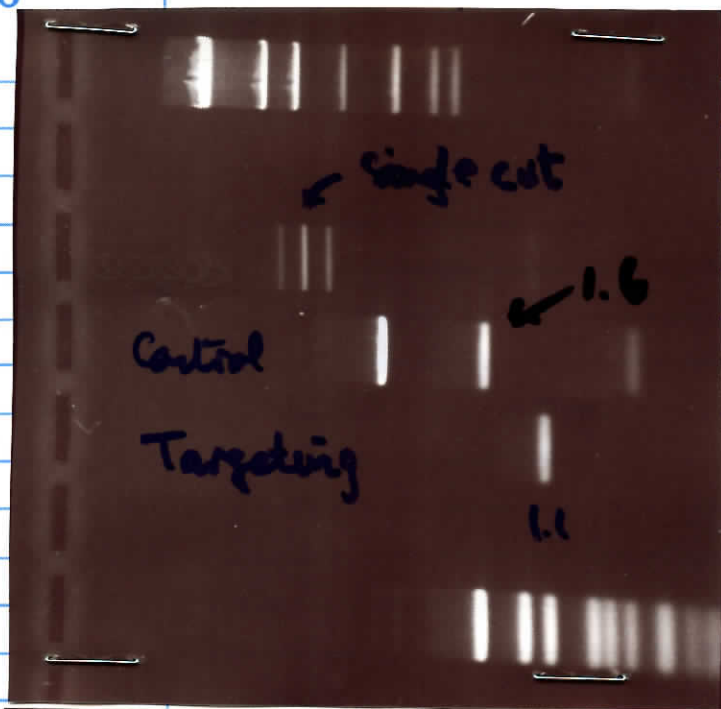
19 μ l $\cdot$ Hx1 6 μ l / 10ml RNAse
 stain

2/10 10:25 am
 2/9 Preparatory

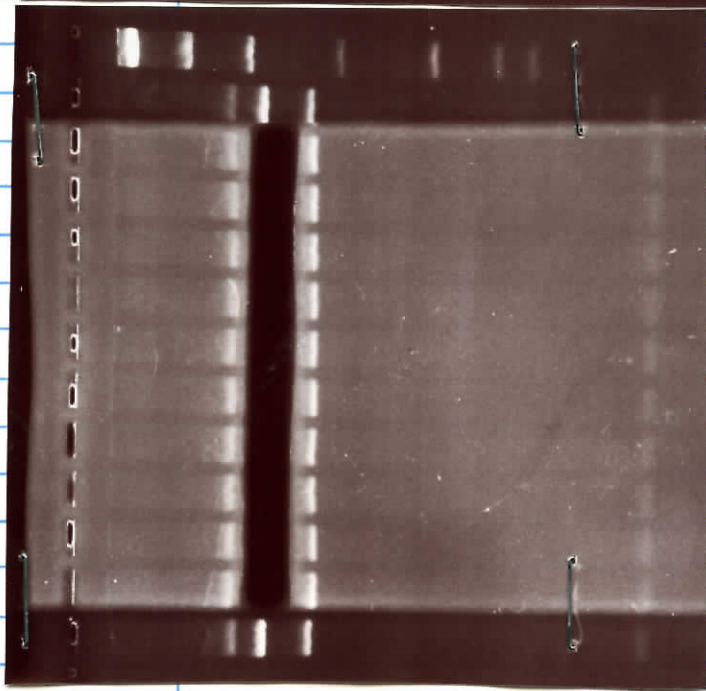
J'5
#⑨ Cla

Recovered all into 80 μ l

J'5 1.1
Cla
Blue
From
⑨



① Conclusion: 1.1 kb Cla from ⑨ is fine. J'9 kind check is good. Asc I single cut can be used at full strength & is okay at this point.



② Conclusion: Nice cut! Back into ~~400~~ 0.001M EDTA as J'7 DD Asc

✓
5
J'
Pos

①

Tue. June 22nd

Single cut Asc I DUPDEL

Take $\checkmark$ 3.7 μ l (10 μ g) ctol. DUPDEL 5 without pol A
 $\checkmark$ +176 μ l ddw
 $\checkmark$ 20 μ l 10 $\times$ RI salts

Prewarm to 37° $\checkmark$

$\checkmark$ +2 μ l (200) Asc I lot 10 (100/ μ l)

37° 6 min $\checkmark$

$\checkmark$ Inactivate at 180° for 13 min.

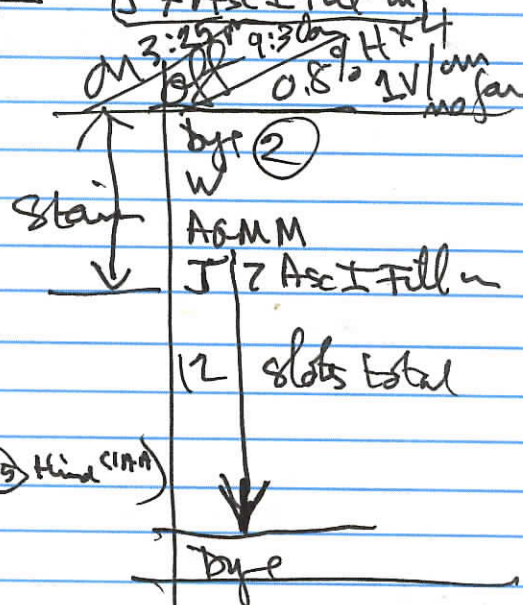
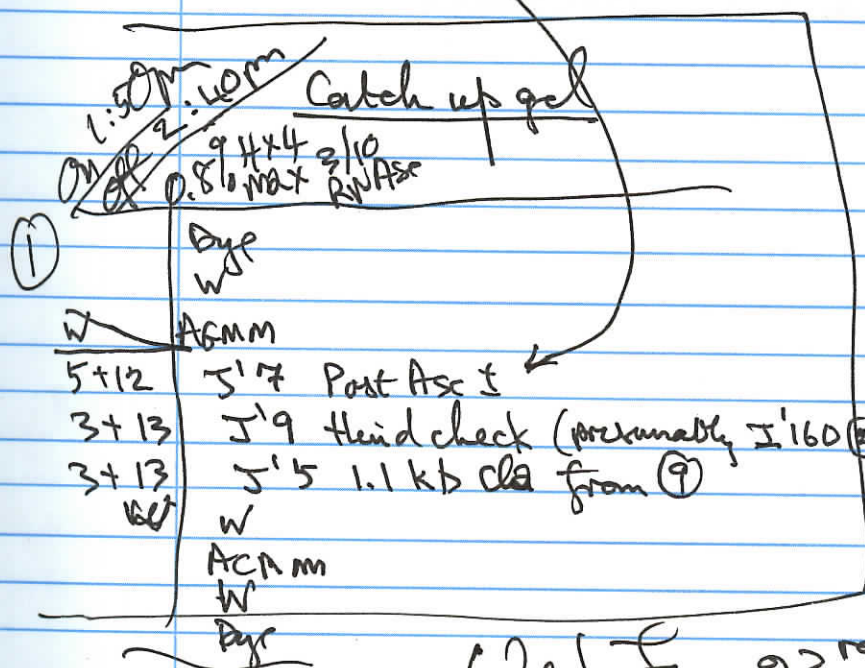
$\checkmark$ +50 μ l γ 3 5 $\times$ sequence buffer

$\checkmark$ +2.5 μ l F193 5 mM dNTPs 60 μ l 100 μ M final

+5 μ l (100) Klenow polymerase lot 3/Dec 00 BM
 37° 1 pm to 3 pm

$\checkmark$ 5 μ l +12
 J'7
 Post Asc

J'7
 Asc I



Wed June 23rd R.W.S. day (20.5)

Take $\checkmark$ 2 μ l 10 $\times$ p95 lig. buffer
 $\checkmark$ 15 μ l ddw
 $\checkmark$ 2 μ l J'7 DDAsc
 $\checkmark$ 2 μ l T4 ligase (dilute) lot 970
 37° 11:45 am to 1:45 pm & to cold.

J'7
 lig.



- ① Condensation. Suspiciously like a poor cutting side J'9 (7) (11) (14) (16) & (18)
are like (18) & (21) Black from J'3 / Re-test J'9 (7) (11) → J'30

Re-test 5'9 (7) (11) $\rightarrow$ 5'3 (1)
diluted after overnight
digestion.

but size is a bit long.

error

)- conceptual error! there is still a cloning Hind III site in the control multi-cloning site. Will need a new strategy.

①

2.45 P
OK 5:27
H25

1.29 P
RM

Day
FG
①
1
J3
Black
(27.5)
(6)
(1)
J9
Black
(27.5)
(20)
J30
Black
ACR
MM

3.27
HX2.5

1.29 Ag
RNAse 3/10
max
6v/u

← closer to
5 v/om catenous.

Tues. June 22nd

Repeat of Hind to Cla for control

Unnecessary
- still have
I'160 (27.5)
Hind III CIAA

Take 20 μ l 10x R1
+ 3 μ l I'143 (27.5) CIAA mini
+ 177 μ l ddw
+ 1 μ l Hind III 37° — to —
1x ϕ H/HOQ, 2x CIAA

J'9
Hind (27.5)

✓
3+13
J'9
Hind
check

also Take 2 μ l I'160 Hind (27.5) CIAA
✓ + 2 μ l 10x p95 buffer
✓ + 13 μ l ddw
✓ + 2 μ l I'156 Hind → Cla/Hind oligo

J'9
ligⁿ-

15⁰⁰ 12 noon to 3:20 pm → 37° 3:20 pm to 4:05
to cold

Transformation

brief for v30m

3/6/97 J'9 FB #446 80 μ l ✓ Plated on 1x Amp NZY
J'9 ligⁿ 5 μ l ✓ R 20, 5, 20, 5/
0⁰⁰ 4:05 to 4:50 pm (10) (6) (4) 16, 5/16
42° 90 sec. 37° ~ 5 pm to 9 pm

Also picked colonies from J'3² (Black) transformation
for test

~~Transformation~~

→ Picked to J'9 mini (1) - (20) 37° 6:10 pm

J'3² processed with 1x ϕ H/HOQ @ 1x CIAA before Propanol. looks OK
J'9 ditto

Tues. June 24th

Take 54 μ l 10x Buffer 4 NEB
54 μ l 10x BSA
421 μ l ddw

Blue
+ J'3¹

11 μ l (55v) Cla I hot 33 (5 v/ μ l)
20 μ l + 1 μ l mini J'3² (1) - (6) & J'9 (1) - (20) 37° 12:50 to 2:24 pm
(27.5) Black

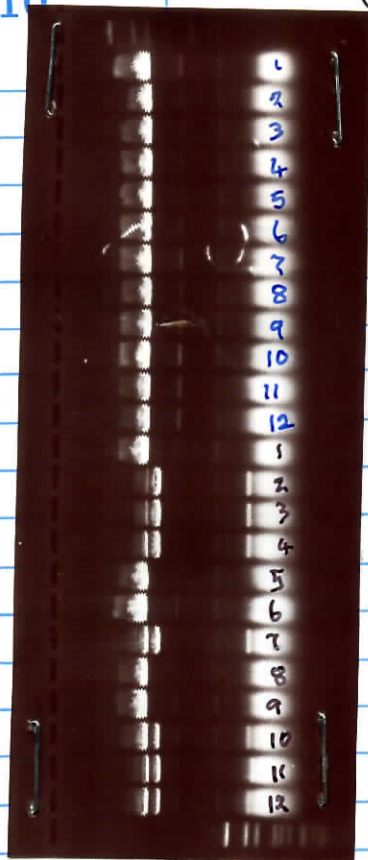
① Conclusion: Excellent yield ~ all have 9 single AscI ~ there appear to be two regular classes & one aberrant.

② 3, 4, 7, 10 & 12 have single ^{AscI} site on ClaI side (HpaI)

1, 5, 6, 8 & 9 have single AscI site on HpaI side (group A).
Aberrant is 11 - some deletion in Neo.

I need AscI on HpaI (Not I) side, ∴ retransform one of each into FB446 for ^{Neo} growth & streak out one of each for ^{Sylvie} growth to grow as they are. Need to retest all when regrown.

Pick ^{Blue} 4 & ^{Black} 5 for streaking → Abandoned
& ^{Blue} 2 & ^{Black} 9 for FB.
← This is the one I need



23?	
1	Dye
2	no
3	AG
1	}
2	
3	
1	}
1	
12	
1	}
2	
1	
12	}
1	
1	
12	Ac
	w
	De

Wed. June 23rd OS. / R.W.S. 7th day!

Asc I sample (cont)

3:30
0.80
Met

Age ①

AGMM

1 Blue
2 Asc I

2 no good

Transformation

12 sh'd have been DH5α!

5'9 FB 446
J'7 high
00 5:31 to 42°

80 pl ✓
5 pl ✓
6:20 in AMP NZY
32° 6:25 pm
9 am - no colonies

2 Thru June 24th

Black
Asc I +
12 Hpa I
ACR MM
W
DYC

J'125 DH5α
J'7 high
✓ 00 1:16 to 2 pm
42° 90 sec

Plated ①
R 20, 5, 20/16, 5/16
11 AMP NZY
37° 2:10 pm
Wet

Fri. June 25th

5'11 Minis ① - ②4 to 11 AMP NZY 37° 1:45 pm to ~ 12 noon

Processed to 50 pl as minis ① - ②4 J'11.

✓ Take 100 pl 10x ~~NEB~~ N&B buffer 4

445 890 pl Ldw

5 10 pl Asc I (50) Lot 10 (100 pl) 20/RX
250 pl 250 pl 5 pl Hpa I (50) Lot 43 (50 pl) likes buffer 4

1 pl Mini ① - ②4

Blue Asc 37° 2:05 pm to 3:20 pm

+1 pl Mini ① - ②4

Black AAT

Tues will distinguish which Asc I site in plasmids with only 1 site

Saved remaining 12 ①3 - ②4 in reserve.



ABANDON

① Conclusion: B/S clg
is fine. The lig.
is bad - too much
internal stuff...
repeat Hind III,
prepare 1.6 kb, ligate
linkers, re-purify
& proceed or redesign
to accept Hind III

Dont forget CLAP on B/S clg

Fri. June 25th - Ext. Hind 1.6 kb

Control construct

- can sequence also.

Since the Cla(ext. Hind) 1.6 kb Hind
fragment cannot be made, I will use
the Cla ← Hind III 1.6 kb → Cla

fragment ~~prepared from I'160 after ligating on oligos~~ prepared from I'160 after ligating on oligos. Can orient with multicloning (internal) sites.

- Take ✓ 100 µl I'160 (27.5) Hind III CIAA
- ✓ + 12 µl 10x P95 lig. buffer
- ✓ + 8 µl I'156 Hind → Cla/Hind oligo
- ✓ + 5 µl T4 ligase (dilute) Lot 970

J'13 oligo lig

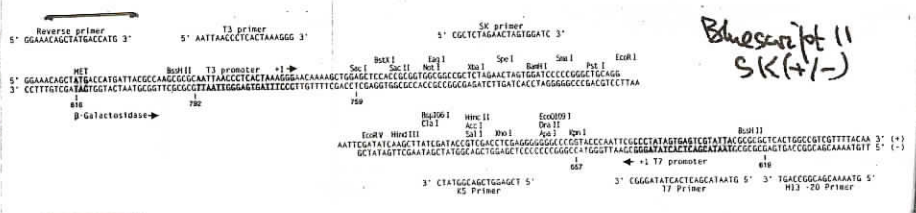
15° 8:35 am to 11:35 am RT, 11:35 am to 12:45 37° 12:45 to 1:45 pm

- Take ✓ 20 µl 10x Buffer 4 NEB
- ✓ 20 µl 10x BSA
- ✓ 160 µl ddw
- ✓ 1 µl (4.5 µg) J'13 BS (Phuescript K5 II)
- ✓ 2 µl (10 u) Cla I Lot 33 (5 µg) T-29 MEH 4.5 µg/µl

J'13 B/SCla

6+12 PreCla

6+12 Post Cla



37° 8:45 am to 11:55 am 75° 30 min

① Dye

PreCla Post Cla

ACRMM

J'13 oligo lig

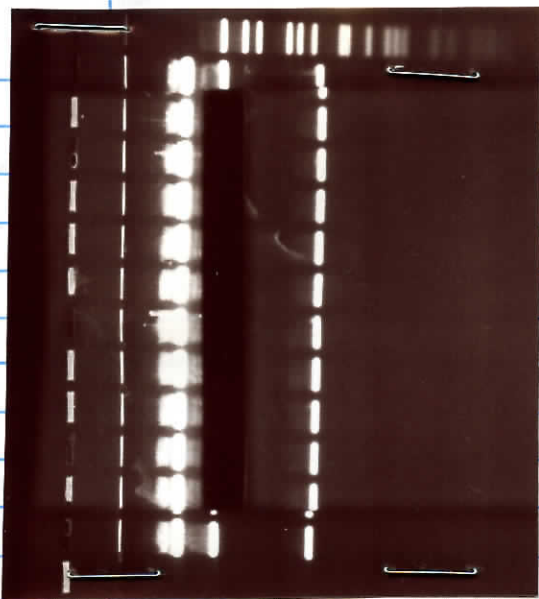
- slots

Prepare

On 2 pm off 4:45 pm

1.29 Hx1 sea plaque RNA prep

6/10

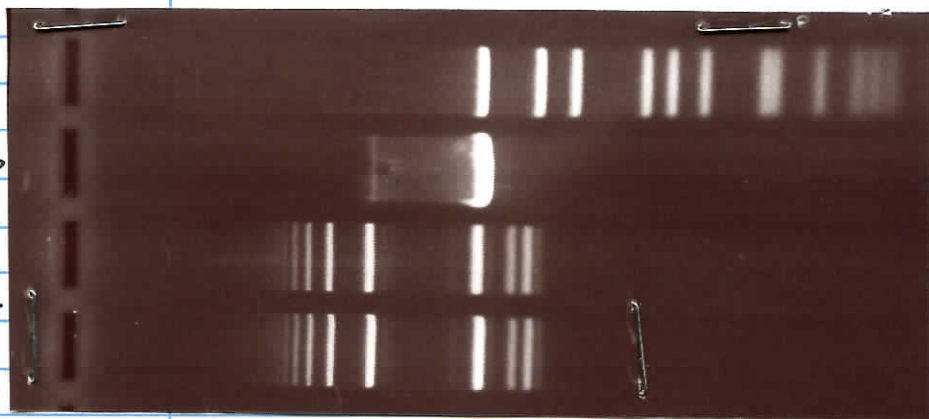


① Nice cut } Recovered into
~~75pl~~ as J'15/1.6kb

1.6kb

Post
 High
 Stem

Post
 Clea

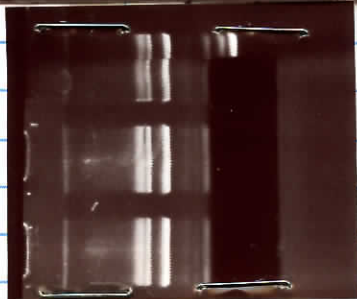


② Promising. Postels
 may be a bit shorter.
 Prepare monomer
 for cloning. Use
 3+13 of J'15 1.6kb
 as cutting guide.
 Don't forget CIAP
 on the TOPDEL ligase
 or B/S



③

1.6kb
 J'15
 H+1
 N6V
 RCRM
 3+13
 1/2 X → J'15 L+K+cl
 slot 2 good slots
 Bye

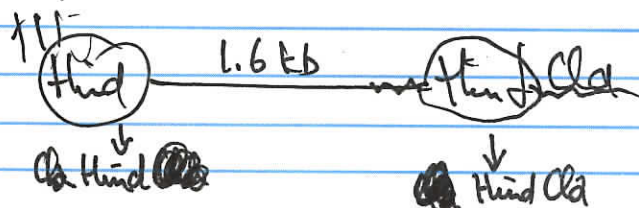


③ Conclusion: Good cut. Note
 the closed circles have
 reversed relative to linear
 in SeaPlaque.
 Recovered into 20pl as

J'14
 cl 1.6
 fragment

Fri. June 25th Hind 1.6 kb Control

Restart on 1.6 kb.



Take ✓ 20 μ l 0.1x RI

✓ 177 μ l ddw

✓ 3 μ l I'143 (27.5) $\text{C}_{14.4}$

✓ 1 μ l Hind III lot 51 (200 μ l)

37° 5:35^{pm} to ~7:30^{pm}

J'15
27.5 Hind

Sat. June 26th

2:55^{pm}

①

Dye

W

ACR MM

Stain

1.2% Hx2.5

Seaplague

J'15 (27.5) Hind

8V/cm

12 slots

Prepⁿ initial

5:55^{pm}

15:59 Hx1

10V/cm

Dye

W

ACR MM

Sat. June 26th

J'15 1.6

J'15 1.6

J'15 1.6

J'15 1.6

J'15 1.6

J'15 1.6

J'15 1.6

J'15 1.6

J'15 1.6

J'15 1.6

J'15 1.6

J'15 1.6

J'15 1.6

J'15 1.6

J'15 1.6

J'15 1.6

J'15 1.6

Take ✓ 50 μ l J'15 1.6 kb

6.4 μ l 10x p95 buffer

✓ + 2.5 μ l I'156 Hind → Cla/Hind oligo

✓ + 5 μ l T4 ligase White lot 970

Cold 5:25^{pm} to 11:50^{am} ~~37° 12:15 to 4:13^{pm}~~

✓ + 8 μ l X19 Kim's 10X Kinase buffer (ex ϕ 121)

✓ + 8 μ l 10mM ATP C'37

✓ + 1 μ l (300) ~~life savers~~ T4 kinase lot 906 Marigat

37° 12:15 to 4:13^{pm}

Will need to cut with Cla I again

✓ + 6.5 μ l 8M NH₄Ac

✓ + 180 μ l G100

Freeze. Cut. Dry. Back into 5 μ l 10x NEB 4

37° 4:55 to 5:50^{pm} → 37° 6^{pm} cold

✓ + 5 μ l 10x BSA

✓ + 40 μ l ddw

✓ + 1 μ l Cla I (50) lot 33 (50 μ l)

Oligo ligation

J'15
Lig-

J'15
HIG/KIN

16 μ l
Post by Kim

16
Post by Cla

16
Post by Cla

16
Post by Cla

Note Sec 127
Final products
have this insert
inverted relative to

GCTCCA
4741 -----
CGAGGT

BclI
aattga
4801 -----
ttaact

TATGcg
4861 -----
ATACgc

cgTCG
4921 -----
gcAGC

Sat. June 26th

Asc I single rate DUP DEL

Transformation		2	9
		Blue	Black
I'139	FB446	80 pl	80 pl ✓
Blue Mini J'11	(2)	1 pl ✓	-
Black Mini J'11	(9)	-	1 pl ✓

0° 6:08 PM to 6:30 PM

42° 70 sec

Plated 10 Amp NZY R, 20, 5, 20/16, 5/16 (2)

as J'17
R (blue) (2) and (black) (9), R, 20, 5, 20/16, 5/16 (9)

37° 6:45 PM

Wet plated - esp. for Blue

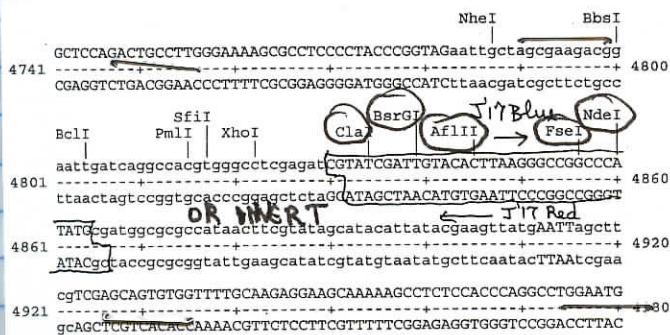
- dried in air hood (sterile).

Black (9) is what I need for that exp't.

Sun June 27th

Best idea is to hold all big growths until the Cla site is changed to a dam methylation insensitive form; can also look for some new enzymes.

Re-map DUP DEL pol A + see : 2



22948	Oligo #	5'	Oligo Name: J'17 BLUE	3'
Date made:		CIGT	ATIC	GIAT
LENGTH: 35		CITT	AAG	GAC
% G+C: 51%		ATA	TIG	
22949	Oligo #	5'	Oligo Name: J'17 RED	3'
Date made:		CIGC	ATIA	TGAC
LENGTH: 35		CCIT	TATA	GTIG
% G+C: 51%		CIGA	TIA	

Sun. June 27th

J'17 2A & 2B Blue

J'17 9A & 9B Black

to 37° at 4:30 PM to 9 AM

1X 004/400 | X CIAA prior to propand.



① Conclusion:- All are as expected
 Proceed with Cla-cut (9A) + (9B)
~~for~~ for insertion of 3
 1.1 Kb insert. Heat inactivated
 the enzymes - 2A, 2B, 9A, 9B Cla
 $\geq 65^{\circ} \geq 20 \text{ min}$ ✓

Mini7

ON

①

Uninhibited

Monday June 28th
Single Asc I (contd.)

Single Asc Dup Dup
Hpa (2)

Take ✓ 75 μ l 10x NEB buffer 4
✓ 75 μ l 10x BSA

✓ 600 μ l ddw

✓ 625 μ l

✓ 125 μ l

+ 12 μ l $\downarrow$ Cla I (80 u)
wt 33 (30 μ l / μ l)

+ 2.4 μ l Asc I (24 u)
wt 10 (100 μ l / μ l)



0.8% HMA 3/10 RNAse Max

① Dye
W

~~AGMM~~

~~2A 2B 9A 9B~~

2A } Cla
2B }
9A }
9B }

2A }
2B }
9A }
9B }

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2B }
9A }
9B }

2A }
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2B }
9A }
9B }

2A }
2B }
9A }
9B }

32° 3:10 pm

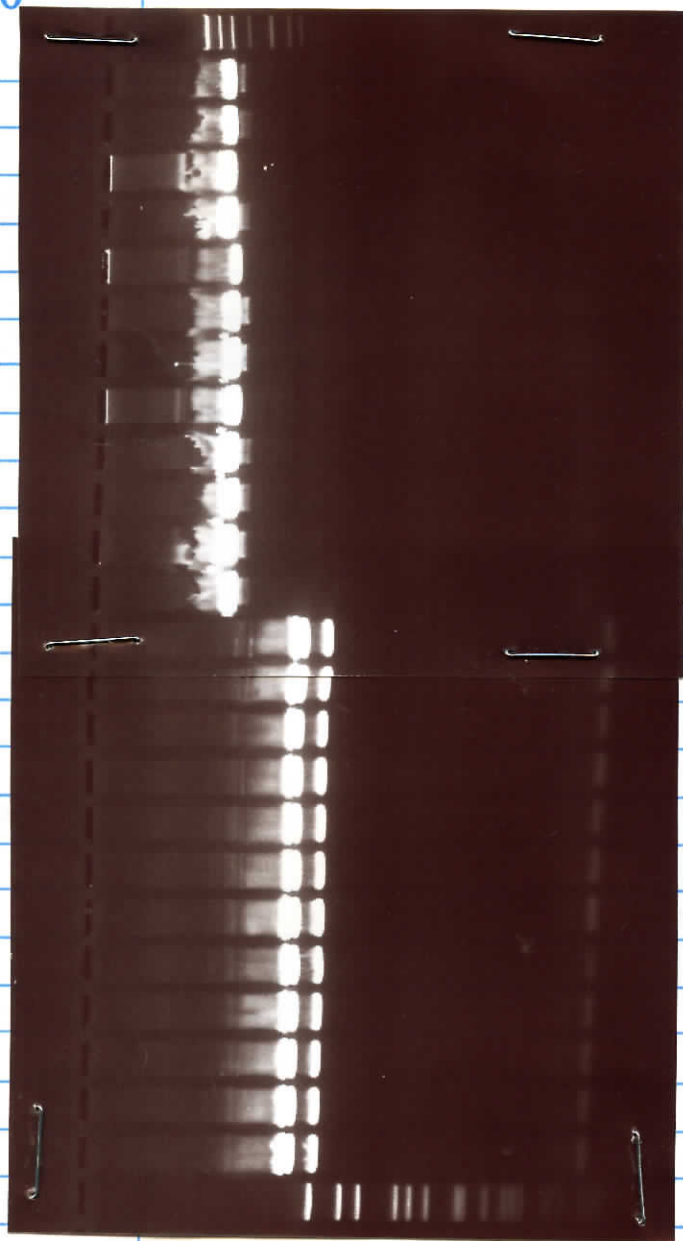
4:15 pm

4:30 pm

Pool 2A+2B as J19 2 DD Cla

Pool 2A+2B as J19 (9) Cla DD after heat inact.

Unaltered



Unchanged
Dup Del
gives one
band with
Hind III -

① Conclusion

Only background
with Targeting
series & little
with control.

Better clean up
the single cut

DUP DEL by using
gel & repeat.

OR - pick 1/8 max
targeting & screen by
size KOAc gels.

Dup Del unchanged gives: -
100 bp, 1.6 kb, 2.2 kb, 2.3 kb
- all obtained
with Eco RV + Bam HI

Picked Y25 - Y72
Blue J21

to 1X Amp NZY at 7pm
Thurs. July 1st.

Thurs. July 1

Take ✓ 26 µl OXRI

✓ 231 µl ddw

✓ 3.3 µl (65v) Hind III (65v)
Lot 51 (20 µl)

1 µl + 20 µl J21 Blue minus (1)-(12)

1 + 20 J21 Black/Pal Mini (13)-(24) Lot 79 (20 µl)

37° 1:10 pm to 3:30 pm

✓ 26 µl OXRI

✓ 228 µl ddw

✓ 3.3 µl (65v) Eco RV

✓ Lot 32 (20 µl)

3.3 µl (65v) Bam HI

Lot 79 (20 µl)

Wed June 30th

J21 (1)-(12) Blue

(C13)-(C24) Black

37° 3:20 pm

1X NZY

AMP

~~1X Amp NZY at 7pm~~

~~Thurs. July 1st.~~

v33

①

w

Ac

T1

T2

i

T12

C1

C1

i

i

C2

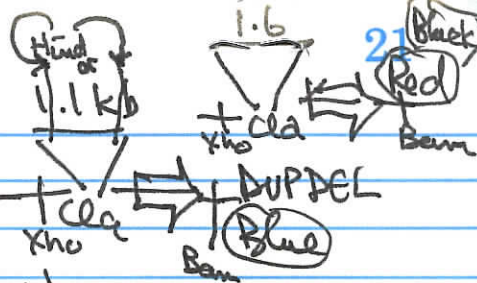
Ac

i

T

23:40
 10/10/10
 10/10/10
 3/10/10

Times June 29th



① Dye
 W
 AGMM

3' homology insert

targeting (blue) + control (red)

T1
 T2
 Blue
 Target

pool (A) + (B) J19 cla heat shock! digests
 as J19/cia DD

T12
 C13
 C14
 EcoRV
 BamHI
 Black
 Control

Take 50 μ l J19/cia DD
 + 12 μ l 5X CIA buffer
 + 2 μ l (2 μ l) CIA had stock
 52° from 9:45am to 10:30am
 1x ϕ OH / 1x OQ

J21
 CIA DD

C26
 ACMM
 W
 Dye

2x CIAA
 + 6 μ l 8M NH₄A + 155 μ l EtOH Freeze Cent. Dry.
 Back up into 30 μ l 0.001M EDTA

J21
 QAP
 CIAA

Didn't change name

Untested

Take 10 μ l J21 QAP DD
 + 10 μ l J5 1.1 cld from ①
 + 2.2 μ l 10x P95 buffer
 + 1 μ l T4 ligase buffer lot 960 1 μ l

10 μ l
 10 μ l J14 cld 1.6
 2.2 μ l
 1 μ l

Transformation	Blue	Red
80 μ l	80 μ l	
5 μ l	5 μ l	
5:35pm	6:03pm	

J21
 T Higⁿ
 Blue

J21
 C Higⁿ
 Red

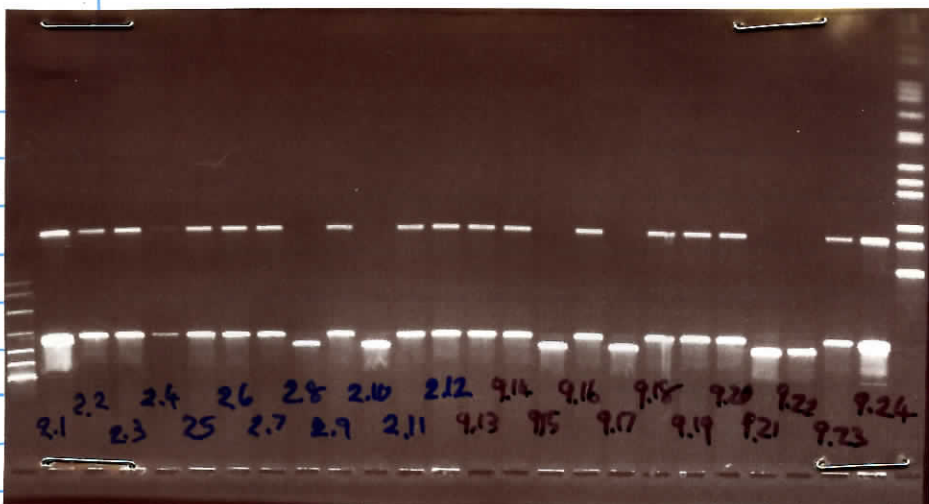
15° 11:30 to 2:40
 RT 2:40 to 1:50
 37° 2:50 to 4:05 → RT → 5:35

J21
 C Pre

Plated J21 Blue
 J21 Red

20 5 20/16, 5/16
 20 5 20/16, 5/16

1x Amp NZY
 37° 6:45pm
 n/a



① Candubia 75% yield. Streaked 2.1 Blue J23
 J23 9.24 Black as good growers for bulk
 sequencing. To 37° 7pm Thurs. July 1st

① 5:37
~~2.1~~ 2.159

HF)
 3/6 R

Dye

W

AG

2.1

2.2

2pl 2:10

Blue

2.12

9.13

9.14

9.24

ACR

1pl

2:17

Dye

Black

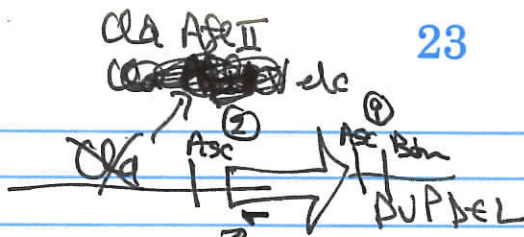
1.5g

3/6 RNasep
10V/c

Tues. June 29d

23

8(2)9 DUPDEL modification



Take 20 μ l J'19 (9) Cla II

2.9 μ l 10x P95 buffer

2 μ l J'17 Blue (linker oligo)

2 μ l J'17 Red (linker oligo)

2 μ l T4 ligase dilute lot 960

5' Neo Red 20 μ l J'19 (2) Cla II

2.9 μ l

2 μ l

2 μ l

2 μ l

15° 10:20^{am} to 2:40 RT 2:40 to 2:50 37° 2:50 to 4:05

to RT to cold

Transformation J'23 Black Red

J'23 Blue

I125 DH5 α
(9) lig.
(2) lig.

80 μ l
5 μ l (9)

80 μ l
5 μ l (2)

0°C 5:00 to 4:20 90 sec

5:50 to 6:20 pm

Plated 1x NZY Amp R, 20, 5, 20/16.5/16 (2)
37° 6:45 pm Blue
Red R, 20, 5, 20/16, 5/14 (9)

J'23 Minis (2)1 — (2)12 Blue
(9)13 — (9)24 Black

1x Amp NZY 37° 3:20 to 9 am

(12) Over Plated

Test with AP II and Bam HI in Eco RI salt

Sequence left with G'143 5' Neo Red.

Take 50 μ l 10x RI salt

440 μ l d.w

13 μ l (130) AP II lot 33 (100 μ l)

6.5 μ l (0.130) Bam HI lot 79 (200 μ l)

37° 4:17 pm
5:17 pm

Tues. June 29th

NPRC

Since we failed to generate an NPRC duplicate,
N.M. suggests moving intracytoplasmic domain of
NPRC into one copy of NPRA. This will reduce
NPRA & increase "NPRC". sh'd be very nicely
hypertrophic. Make that my next project.

Sequence data confirm (1) has 1 AseI on Bam side
Not
(2) has 1 AseI on Xho side

All insert sequences are okay. Both are
oriented in same direction - opposite
from J17 diagram.

Fri. July 2nd

DUP DEL
mod.

QA $\rightarrow$ Not
27
(5' Neo Red V58)

Sequencing DUP DEL modifications

Re-name J'2.1 Blue

OSDUPDEL 2

J'9.24 Red

OSDUPDEL 9

Sequence both with the two universal primers:

G'143 5' Neo Red for group B sites
and V58 Red G'135 for group A sites

Submitted
July 2

Seq 118

14 pl ✓

G'143 5' Neo Red
✓ 5 pl 100

OSDD (2) 1 pl

Minis from J'23

Seq 119

14 pl ✓

G'135 V58 Red
✓ 5 pl 100

OSDD (2) 1 pl

(2) (2:1)

Seq 120

14 pl ✓

5' Neo Red
✓ 5 pl 100

OSDD (9) 1 pl

(9) (9:24)

Seq 121

14 pl ✓

V58 Red
✓ 5 pl 100

OSDD (9) 1 pl

Good
sequences

Template

J'23 2.1 Blue now called
" 9.24 Red " "

OSDD 2

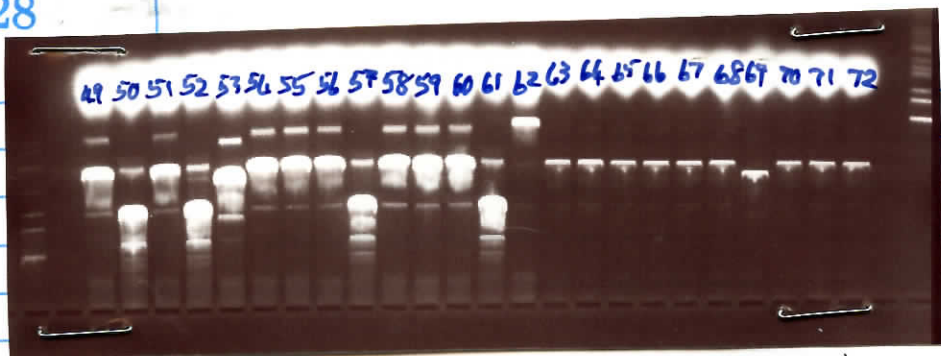
J'23

OSDUPDEL 2

OSDUPDEL 9

OSDD 9

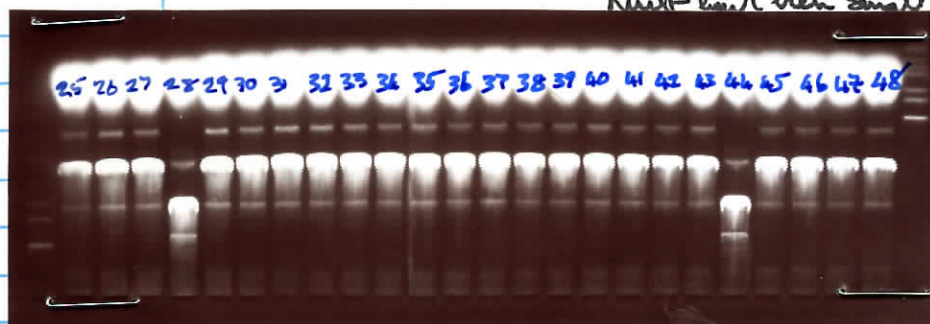
J'23



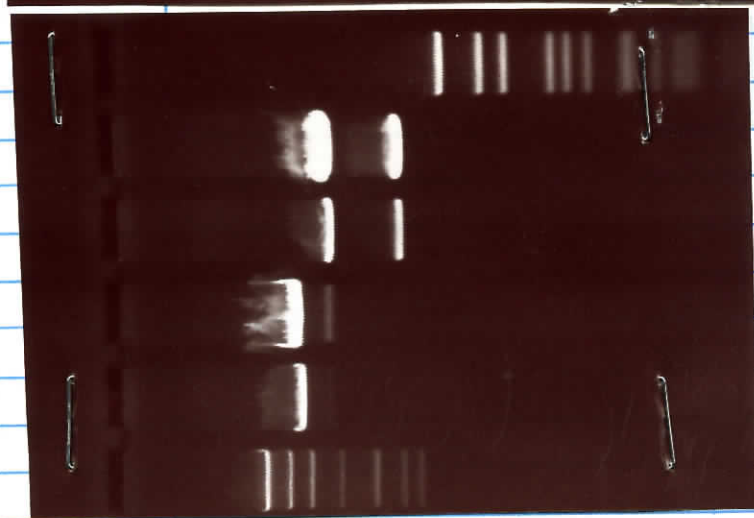
① Conclusion

50, 52, 57 & 61 are
some type of dimer
(53) & (69) could be
correct. Continue

must have been small colonies.



② 28 & 44 dimer.
No 1.1 inserts.



③ Conclusion

Both (53) & (69) are the

same. The
orientation is ~~incorrect~~
incorrect, and
but either

Clal or Pme I
did not cut.

Test again with
Clal & Hpa II
(now found)

Meanwhile scan
another 48.

Fri July 2nd

12:35pm
Off 1:45pm
0.8g HxH
NoX

Dye
AGM
W

~~CLR~~ CLR 3' homologous insert

(49)
(57)

Mini J'21 (49) - (72) processed to KOAc by 12:30pm

(70)
(72)

Mini J'21 (25) - (48) - - - - -

(70)
(72)

J'21
minis

W
PCR

Talce 4.4µl 10x NEB buffer 4

4.4µl 10x BSA

✓ 33 µl ddw

2 µl Cla I (10U) hot 33 (50/µl)

Rest

2

Off 1:45pm
On 2:50pm
Lilko

Dye
W
AGM

(25)
(28)

3

Off 5:35pm
On 6:00pm
4.5g HxH
3/10RMHx

Dye
W

(53) H(ind III)
(69) H(ind III)

(53) CP
(69) CP

AGMM
W

Dye

1 µl J'21 (53)

CP + 2 µl Pme I

37° 3:40pm

✓ 4.4µl 10x RIsalt

✓ 39 µl ddw

1 µl Hind III (20U) hot 51 (40/µl)

20

Rest

20

1 µl J'21 (53)

H

37° 3:40pm

5:20pm

2 µl J'21 (69)

CP + 2 µl Pme I

37° 3:40pm

✓ 4.4µl 10x RIsalt

✓ 39 µl ddw

1 µl Hind III (20U) hot 51 (40/µl)

20

Rest

20

2 µl J'21 (69)

H

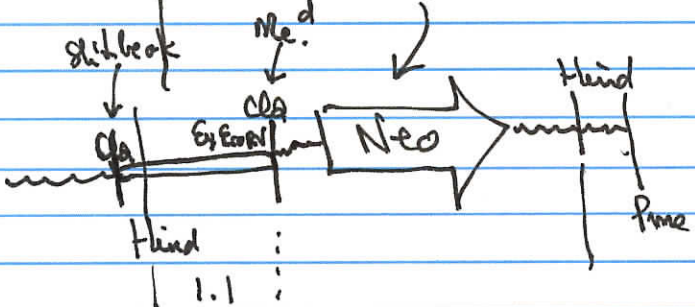
37° 3:40pm

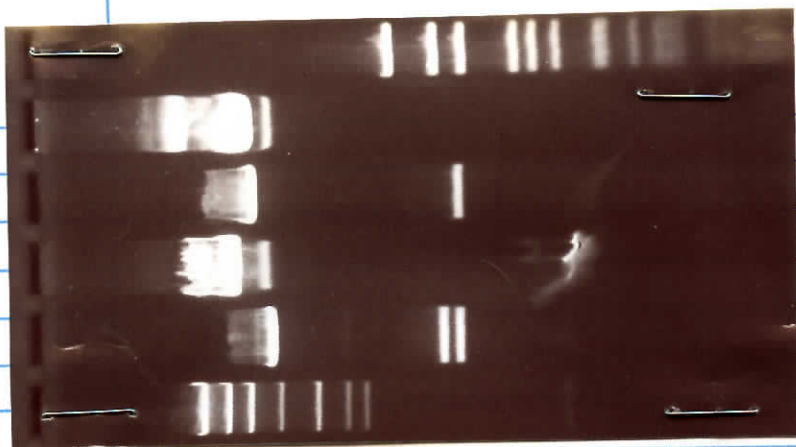
5:20pm

Forget that no of
Cla I sites will
be med,
Hpa II exhausted,
Pme I uses buffers 4.

See I'153

Wrong
orientation





① Conclusion:

Both cl sites are not cut in 53; both are cut in 53 Dam.

Hpa I has one site in 53 & this site is present in 53 Dam.

Possibly the cl I

is missing 53.

Since this clone is clearly correct fragment in the wrong orientation it is worth screening more of J21 colonies. Meanwhile repeat the J21 targeting lig. with less DVPDEL.

Can't find any remaining plates!

on 3/10/2

+
5
5
5
5
5
+
+
+

3:03 PM
3:28 PM
1.56
3/10
10/10

Fri. July 2nd

Dye ①

W

ACR

53

53D-

53

53D-

AGM

W

Dye

(53) to Damm - to allow ③ confirm

Take ~~46~~ 46 I'139

80 μ l

1 μ l

J'21 mini (53)

0° 6:59 PM to 7:20

42° 90 sec

Plated

Rest, 20, 20, 20

25 μ l (50) (25)

16, 5, 16

(7) (4)

{May be a short cut} 20 μ l to 10 ml 1X NZY with 2X Amp.

37° 7:30 pm
Nico growth by 9 am - look good!
Proceed to 50 μ l at J'31 (53D-)
with ① H/40 Q at K0 A c

Take 10 μ l 10X NEB Buffer #4

110 μ l 10X BSA

80 μ l J'21

+4 μ l (200) clal Lot 33 (100 μ l)

59

+2 μ l J'21

T (53)

53C

20 + 1 μ l Hpa I

53CH

Lot 43 (50 μ l)

53D-

53DCH

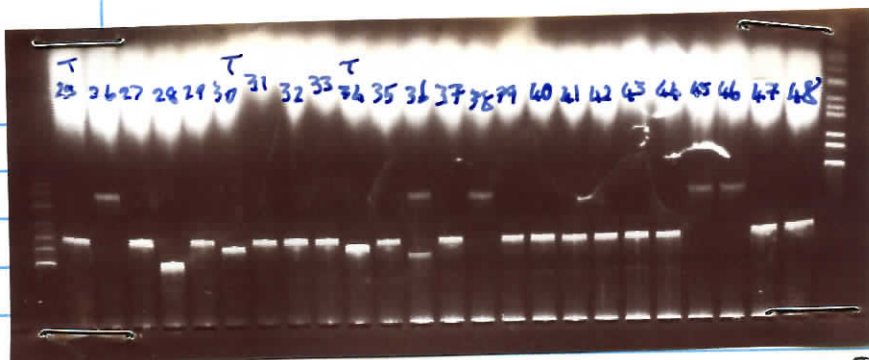
20 + 1 μ l Hpa I

53DCH

37° 10:16 am

2:50 pm

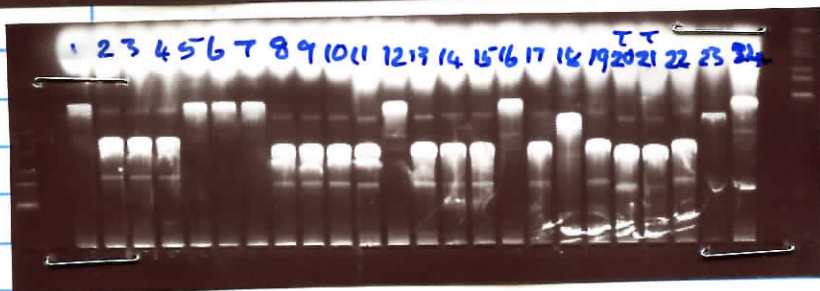
4.4 μ l 10X RT cally
36 μ l J'21
+ 1 μ l (200) Hind III



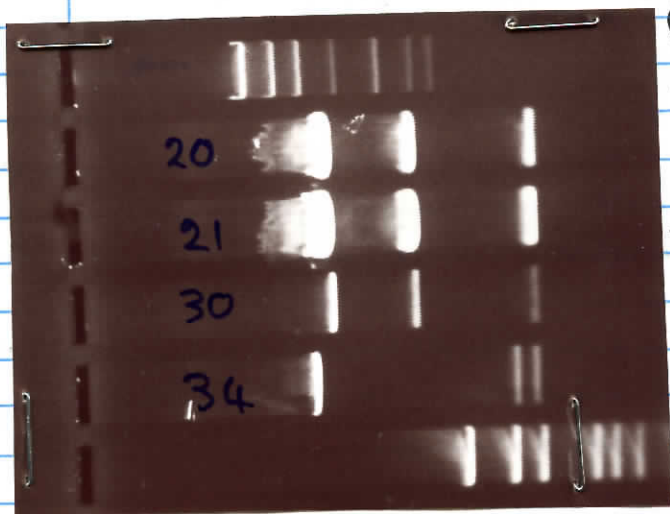
① Conclusion:

17H is fine for superiorly
 (30)(34) are possible.

? 20
 ? 21

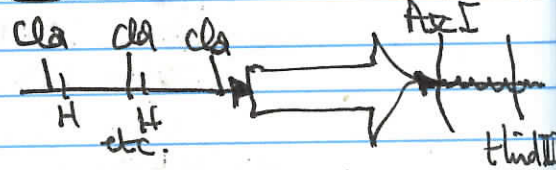


② Conclusion: (21) & (21)
 look good. Use HxI
 in future for KOAc mini's
 (2)(3)(4) look short.



③ Conclusion:

~~(2)(3)(4) look short~~
~~(2)(3)(4) look short~~
 looks as if (20) (21) & (30) all
 are a dimer insertion ~~(2)(3)(4)~~
 & (3)(4) is correct orientation but a dimer



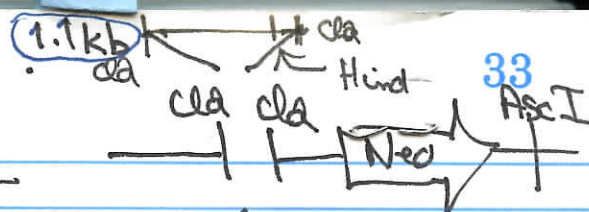
Better process ~~(2)(3)(4)~~

(2)(3)(4) & (8)(9) & (10)(11) etc.

? DD

? correct

① 2 1/2 hr 9 Hx4 ← Error Prob. 4 (X)
0.5 MAX



Sat. July 3rd

Repeat 5' homology assembly

Dye
w
ACMM
(25) J33 min KOAc
(48) J33 T LIG N
ACRMM
w
Dye

Take 1 µl J21 D3 CIAF CIAA (1/10th of previous)
+ 5 µl J5 1.1kb ClaI from ⑨ (1/2 of previous)
+ 1 µl p95 10X lig. buffer
+ 2 µl ddw
+ 1 µl T4 ligase (dilute) lot 970
Cold 5 pm — 5:40 pm Sun.

Sunday July 4th
Independence Day

② 1:23 J21 DH5α
12:28 J33 T LIG N
0.5 Hx4
0.5

120 µl
6 µl
5:47 to 6:40

Dye
ACMM
w
J33
Mini T ① — T ④⑧
J33 min

Plated R, 3x20, 5, 20/16, 5/16 37° 6:50 pm
1x Amp NZY
37° Mar July 5th (740) ⑨ ④
3:20 pm — 8:50 am (25) — (48)
① — ②④

② KOAc Tues July 6th

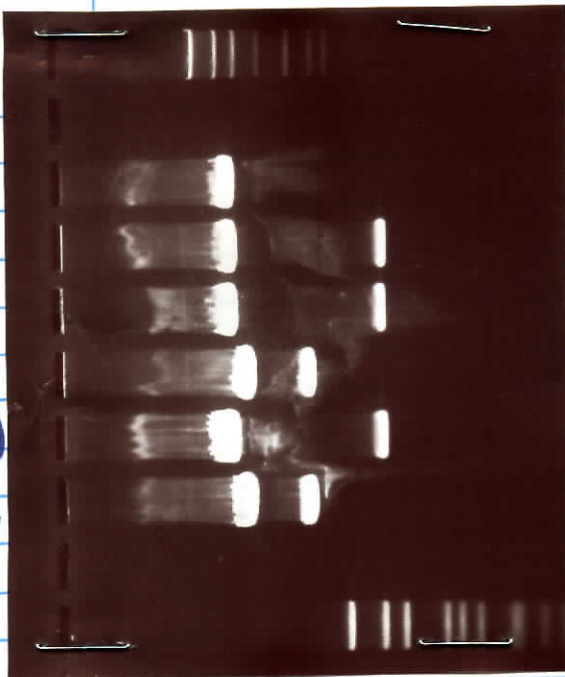
w
ACR
Dye
(3) J33 min
0.5 Hx4
0.5

Take 10 µl 10x R1 salts
90 µl ddw
1 µl Hind III (20U) lot 51 (20 µl)

Dye
w
ACMM
1 µl T 20
1.5 µl T 21
2 µl T 30
2 µl T 34
ACRMM
w
Dye

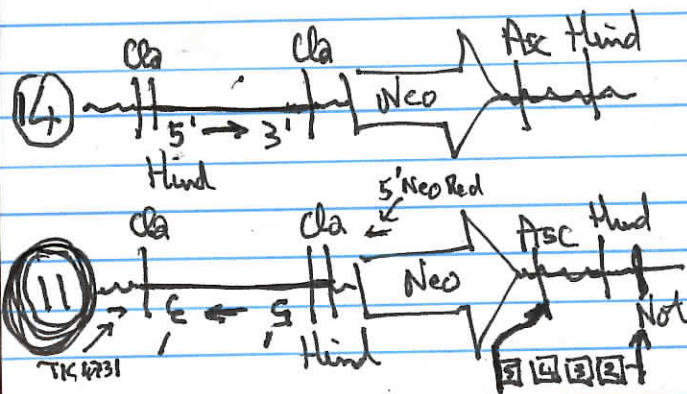
+ 1 µl Mini T ③④
2 µl 2 µl
37° 1:20 to 4:15
②⑤ T ②① ← 90 µl by mistake
1 µl 1.8 µl 4:15 to 5:15

2
8
9
10
11
14

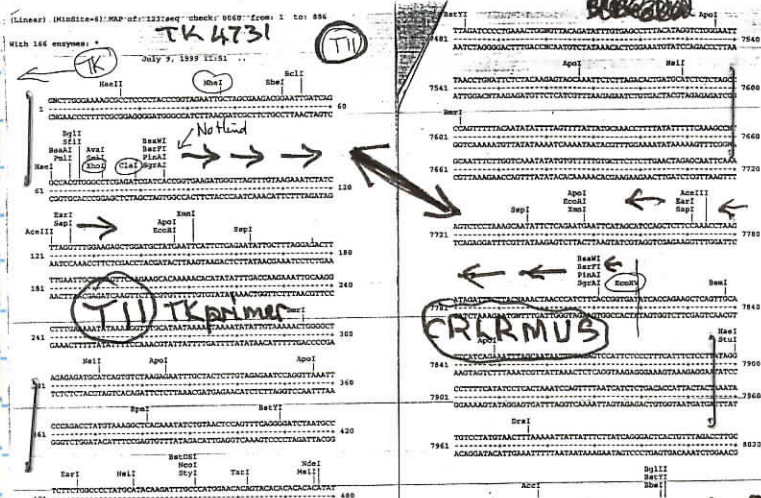
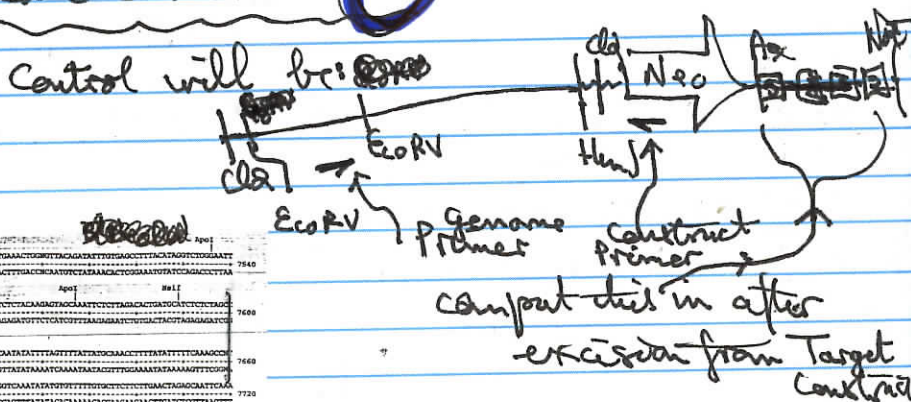


① Conclusion: Good, the detection was correct.

Can use ⑪ as one orientation
→ ⑭ as the other



Since exons ①...② will be inserted into AscI/NotI
→ the derived orientation is **⑪** ←



and Neo end of T14

The sequence from TK end of T11 matches with CRLRMUS
starting at 7819 which is the EcoRV site. This is correct.
The control sequences will start from Bam site at 8350

① 9:45am
 ON 10:10am
 1.29 Hx1
 9.0V/om
 3/10 RNAP

Wed. July 7th
 Target 5' (contd.)

Dye
 ABM
 W

T(2)

(8)

(9)

(10)

(11)

(14)

W

PCR

Dye

B

Take

J33
 Hind III

14µl 0.8 EcoRI salts

12µl ddH₂O

2µl (40U) Hind III hot 51 (20U/µl)

20µl

+ 1µl

J33 mini

T(2)

(8)

(9)

(10)

(11)

(14)

8:22am

to 9:30am

8:32am

	Water	Primer	Template	
Seq 122	14µl	5' NeoRed 100 5µl	J33 Mini T11	1µl
Seq 123	14µl	5' NeoRed 100 5µl	J33 Mini T11	1µl
Seq 124	14µl	5' NeoRed 100 5µl	J33 Mini T14	1µl

Submitted today

Good sequences

Matches Seq 125 (C4)

← As a safety check

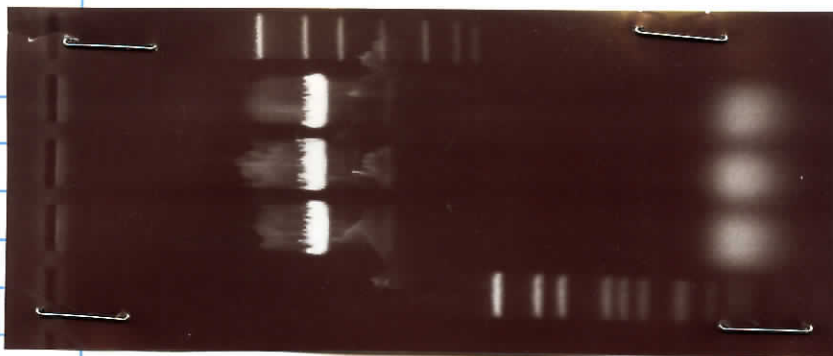
Wait for sequences & then AscI + NotI to purify & assemble the 8' to 2' 483bp fragments into final (hopefully).

Compare T11 with C4 & C10

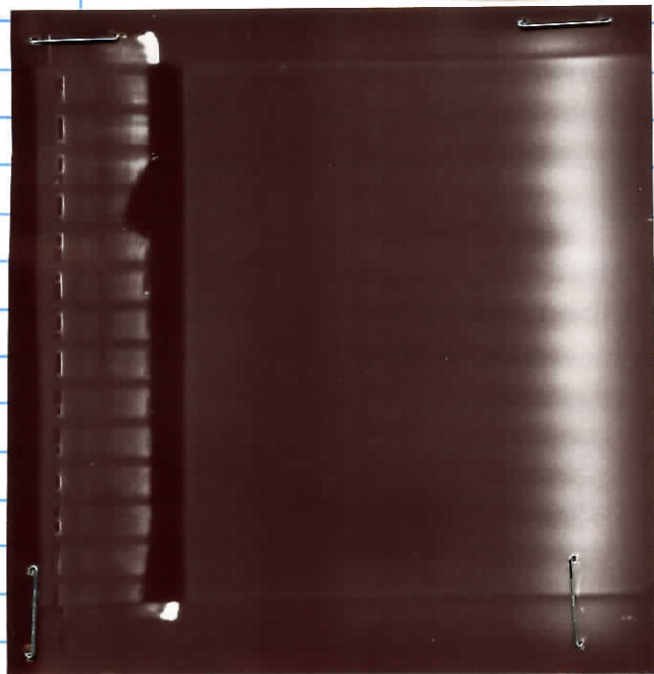
Sequences confirm T11 & T14 are inverted.

Confirmed Hind site in 11 & next to Neo

(contd.)



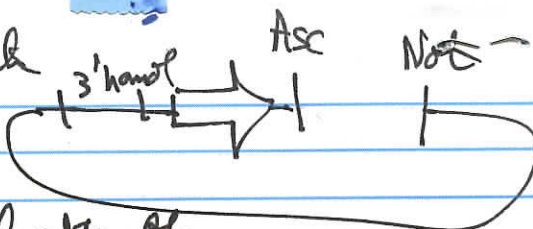
① ^{retrograde site}
All _h cuts look good.



② All gel cuts look good

2/8/0
J3
T11

Fri. July 4th



Preparation of vector

+ 3' handle for final assembly

ON 1:20 pm
0.8% Seap.
Hx1

Take 800 μ l of J'33 mini T11
✓ 30 μ l of 10X NEB buffer 4
✓ 280 μ l ddw

J'39 Asc
T11 Not

Dye
W
AGMM
Post Not
Post Asc
Post Asc+Not
ACRMM
W
Dye

300 μ l
+ 8 μ l (80 u) Asc
37° 9:06 am to 11:15
20 μ l
+ 2 μ l 10X R1 salts
✓ + $\frac{1}{2}$ μ l Not I (5 u) Lot 48
(100 μ l)
37° 9:06 to 11:15
(Chilled)
(Frozen)
Post Not

+ 29 μ l 10X RT salts
+ 8 μ l (80 u) Not I
Lot 48 (100 μ l)
37° 11:25 am to 1:05 pm
Post Asc
Not

20 μ l
Post Asc
(Frozen)

ON 5:04 pm
0.8% Seap.
Hx1

(2)

12 slots
J'39
Asc
T11 Not

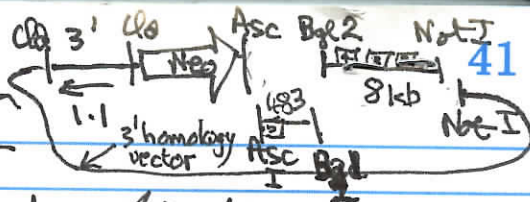
Dye
W
0.8% Seap.
Hx1
W
Dye

Recovered into 100 μ l

as J'39 3' handle
vector

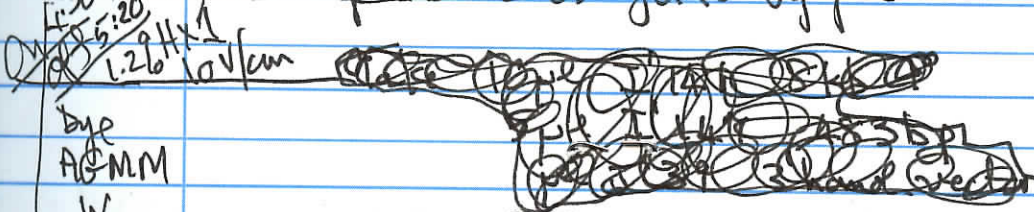
Amount
to 7 kb

Sat. July 10th



First try at final assembly target construct

1. Assess ~~quant~~ conc. first by gel.



Dye
ACRMM

W

I'41 8kb 1° (undiluted)

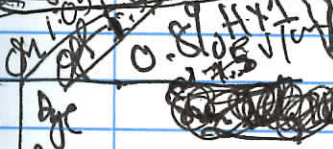
J'39 3' homology vector (undiluted)

I'147 483 bp (undiluted)

W

ACRMM

Dye



ACRMM

W

Pre lig

Post lig

W

ACRMM

Dye

Sunday July 11th

Take 3 µl J'39 3' homology vector

+ 30 µl 8kb 1° I'141

+ 10 µl 483kb I'147

+ 43 µl 8M NH₄ Ac

+ 118 µl EtOH

Freeze Cent. Dry. Back into

15 µl 1x p95 buffer

+ 1.2 µl T4 ligase hot 970

Cold 6:45 pm to 12:45 pm

Cold

J'41 PRE

Transformation

J'21 DH5α

J'41 lig

0° 3pm to 3:30pm

42° 90sec

Plated 1x Amp NZY R, 20, 5, 20, 5

37° 7pm

16, 16

260, 25

J'41 (T19)

37° 11:45am July 14th

streaked out to K.C.

Mon July 12th

Minis J'41 (T1) - (T24)

1x Amp NZY 6:32^{am} 5:40^{pm} 9am

10:35am
11:05am

Dye
W
ACRMM

T1

T2

1

1

1

T23

T24

ACR

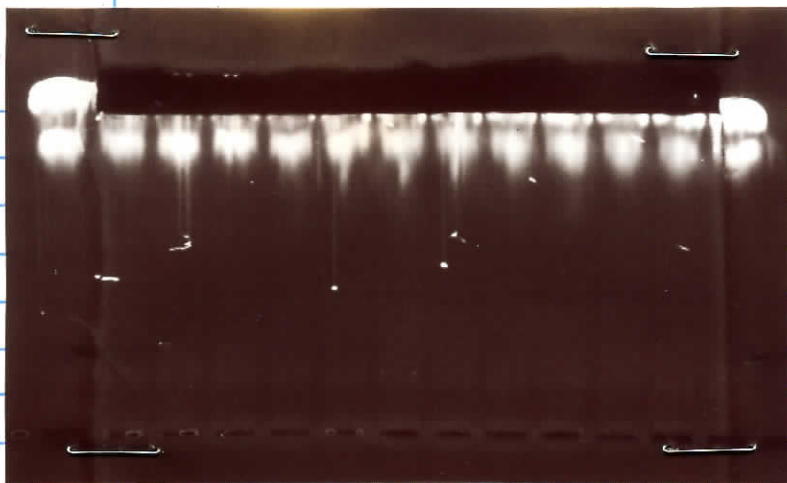
W

Dye

0.60

1x1

10/1cm



① Good cut, but wrong gel!



② ~60% success likely.

Process

C10 C12 C13 C17 C19 C21

Wed July 14th

Transformation

J'21 DH5α

80 μl ✓

J'43 C lig

3 μl ✓

0° ~7pm

7:50pm

42°

70 sec

1x Amp NZY Plated R

20, 5, 20/16, 5/16 37° 8pm

Thurs July 15th

4:50pm J'43 (C) - (C24)

37° 1x Amp NZY 24 min.

Processed to KOA (centrifuge problems)

Mon

July 19th



Preparation for control construct

Take ~~320 µl~~ 32 µl 10x NEB buffer 4
✓ 280 µl Ld w
+ 8 µl (800) Asc I hot to (100/µl)

J'43
(C4) Asc Not
Red

+ 8 µl J'35
(C4)

200 µl
160 µl + 4 µl J'35
(C10) J'43 Asc Not

37° 9:05 to 11:05

+ 33 µl 10x RT salts
+ 8 µl (400) Not I hot 48
at 100/µl

+ 17 µl 10x RT salts
+ 4 µl Not I

0.8% Hx1 - 37° 11:10 to 5pm
1v/cm.

J'43
(C4) Port Asc

13 slots

Error - ~~regular~~
used regular agarose.

Excellent cut however - tried recovery
with (i) phenol (ii) freeze-thaw - cent.
(iii) recovered aqueous phase of ~60% vol. of gel.
2x CIAA, EtoH etc. into 80 µl at J'43 C

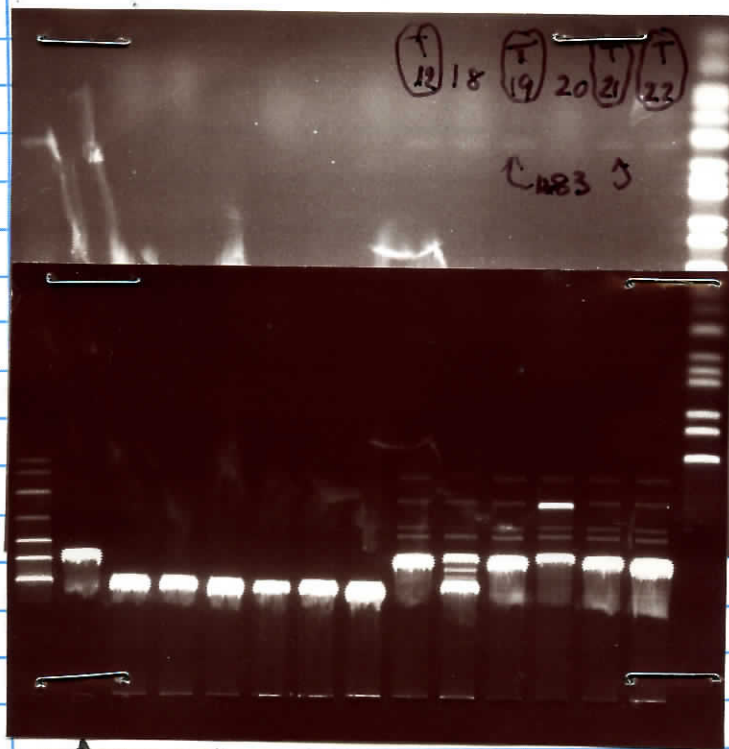
Fine recovery see J'45

Wed. July 14th

Take 5 µl J'43C (3' homologous vector) / 10 ✓
10 µl 8 kb I¹⁴¹ ✓
5 µl 483 bp I¹⁴⁷ ✓
+ 2 µl SM NtH₄A ✓
+ 55 µl EtoH F.C.D. block up into 10 µl 1x p95

J'43
CLIGN

15:00 to 3:05 pm RT 3:10 to 5:20 37° 5:20 to 7pm
+ 1 µl T4 ligase
to cold



① conclusions
 (T12) (T19) (T2) & (T22)
 look okay - but
 need to see the
 Bgl2 - Ase1 483bp
 comes up after RNase,
 even though faded.
 Sequence check.
 Use (T19)
 Recheck when doing
 control.

lrmus_seq x 128.seq July 18, 1999 19:06

(T19) 100% agreement with Bam sequence from 5' end of gene upstream of exon 1.

```

776 CCGTNNNGNANNNNNCATNTCCNCCTTNNNAGTCTTTNTNACCGGCCT 825
15 CGCGTTGCGCGATTTCATTAAATGCAGCGAGCTCGAATTGCGGCC..... 57
826 TTTNATXXXXXXXGGATCCATTATTATGAAATCAACAATACCTGAT 875
58 ..GCTCTAGAACTAGTGGATCCATTATTATGAAATCAACAATACTTGAT 105
876 AATTTTTTTTATAAAAAAGAAATAAGTAAATGCTACTGAGCTACTAATT 925
106 AATTTTTTTTATAAAAAAGAAATAAGTAAATGCTACTGAGCTACTAATT 155
1426 TATTAGATATTTCTTTATTACAGTTCAAAATATTATCCCTTTTCTGGG 1475
656 TATTAGATATTTCTTTA..TTACAGTTCAAAATATTATCCCTTTTC..CGG 703
1476 TTNCCCTNCAAAAAACCCCTACCCCTACC..TTTNTCCCTNCCCTGNTNA 1523
704 TTCCCCCTNTINCAACCCCTACCCCTANCCGTTCTCCCTNCCCTGGTNA 753
1524 CC..ACCCACCATTCCTGTTCTGNCCTGCATTCCCTAGACGGGCGCATAG 1572
754 CCAACCCANCCATCCTGCTTCTGNCCTGGCATCCCTNACTGGGCATAG 803
1573 ACCTTACAGGACC..AGGCCTTCTCCATGANG..CCACT..NACATCCTTGG 1619
804 AACCTNANGGACCAAGGCCTTCTCCATGATGCCACTAGACATCTTGN 853
1620 TCATTGCAG..TGGNCNTGAANCCCTNTTTTTTTGNGGGG 1658
854 TCCTTCCGCTGGGCTGANNCCNNTGTTTTTTTGGTGG 893
  
```

(T19) 129.seq Reverse

```

771 ANGTTGCTCINNAGAGCCNCAAGCCTGCGGCCCTCATTTTATTTCCCTCCC 722
332 ATGTTACCAGAAGATCATGCA.....GGACCCCATTCACAGCAGAAG 375
721 GGCCTTATGCAAAAGG..CCTGGACGGA..GGCTATGCTGGAATG..CGGT 676
376 GCTTTACTGCAATAGGACCTCGACCGATGCTATGCTGGAATGACGTT 425
675 GCAGCAGGG..CGGAATCAATGCAAAAGG..GGCTATGCTGGAATGACGTT 627
426 GCAGCAGGGACGGAATCAATGCAATGCAATGCAATGCAATGCAATGCAAT 474
626 TTGTCCTTCAG 615 ← (T29)
475 TTGATCCTTCAG 486 ← (T29)
  
```

5588 CTCTCCATGTGCTTTTAAAGTAGAATCTATTAATTGAATTTATCTAA 5637
 279 CTCTCCATGTGCTTTTAAAGTAGAATCTATTAATTGAATTTATCTAA 230
 5638 TATATATGTAGATTTTATAGATACAGGGTTTCAATCCATTTTCTTAC 5687
 229 TATATATGTAGATTTTATAGATACAGGGTTTCAATCCATTTTCTTAC 180
 5688 CACACAATTATAGACAGCCCTGAATCTGTTCTACCTAACTATAATTGGAC 5737
 179 CACACAATTATAGACAGCCCTGAATCTGTTCTACCTAACTATAATTGGAC 130
 5738 ATGGATTATCTATTGCATCTCTGATC 5753
 129 ATGGATTATCTATTGCATCTCTGATC 104

Exon 5

AseI

lox

Neo

Bgl2 to Ase1

129.seq shows that there was a Bgl2 site in intron 3, which messes my construct. Exon 3 and exon 6 are now around the Neo & are in same splice frame. But exon 3 can splice to exon 5 (partly deleted) & give useless product. Splicing 3 to 6 is also out of frame out of frame

3bp
(A8-)

Checks on final T construct
2C 3' homology vector

Take $\sqrt{12 \mu\text{e}}$ 10x NEB buffer 4
 $\sqrt{105 \mu\text{e}}$ 50x

✓ 3 μ L (30 μ g) Asc I hot 10 (100 μ L)

↓ 19 pl + 1 pl each of 5' 41 min (T12) (T18 - T22)

37° 2:40 ^{PM} to 4:30 ^{PM} when 10 pl samples were taken to

① 1.1 pl each ~~cell~~ plus 1.2 pl x Eco R1 salt containing 50 Rg2 / 20 pl
On 1.2 pl Hx1
37° 4:35 to 7:05 pm

✓ 12 μ l 10x R1 salts

✓ 102 plddw

3 pe (300) 2 lot 35
(100/pe)

3 pl (30v) Net 1 Lot 48
(10v/pl)

Emor - forgot
there is no pot
plate

Target RNAse I

tailed

Water

Primer

Template

(Seq 127)

14pl

F'103
Blue E/100
5pl

J'41 (T19)
1pl ✓

Seq 128

14pl

~~G/135 V58R~~
~~5 pr~~ →

Jan 19
192

NRM used (30)

Seq 12

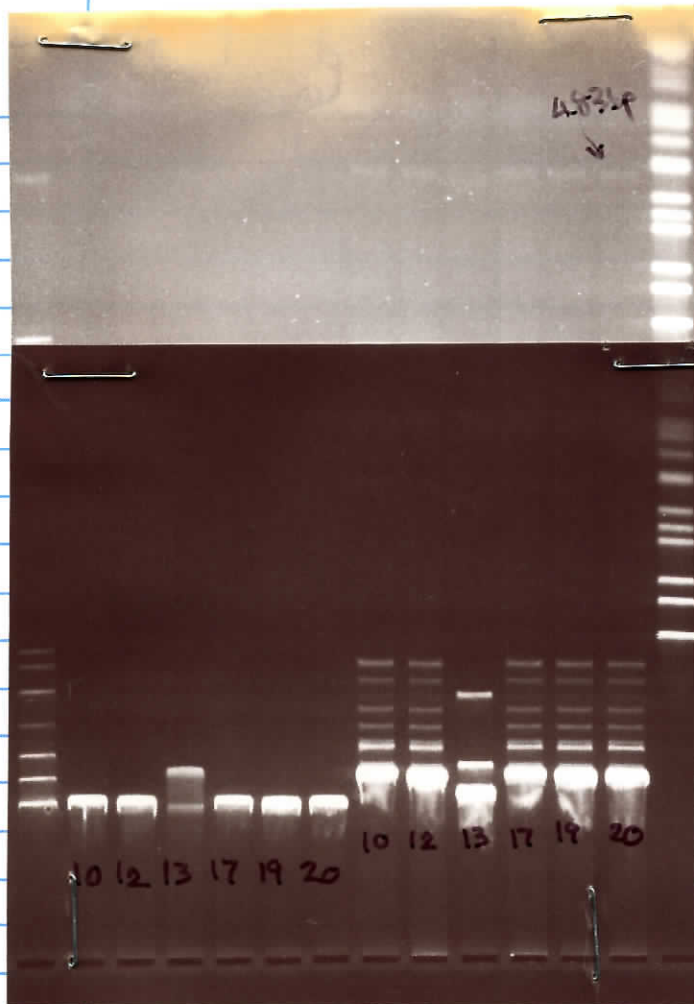
14 p

F'155 ✓
Neo Blue/10
5pe

141 (11)
122 v

Good Sequence

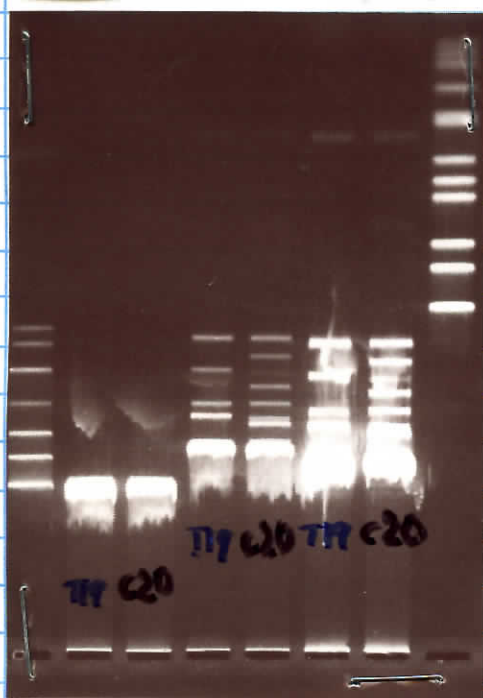
Submitted
July 16th



① Conclusion (C10) (C12) (C17) (C19) & (C20)

are all correct
 streak about (C20) →
 use for sequence checks.
 No Acc I in (C13) 37° 8 pm Fri

② a lot of * activity
 with Acc I under
 RT conditions — the extra
 bands are constant in
 all the clones. ~~later~~
 concluded to be Bgl 2 cuts
 in Dmp del. 483 bp is
 faint — the Bgl 2 digest
 is probably incomplete



② Conclusion: the extra bands
 are not an Acc I problem.
 (C20) is slightly bigger than (T19)

Repeat with new batches of
 Bgl 2 & Not I — latter
 looks OK at 5'38 & 5'43.

Re-run T19 and C20
 0.8% gel overnight.
 Look for resolution
 of the two fragments

(C20)

10V/cm
1.294 x 1
RNAse
3/10

Fri. July 16th
Control assembly (cont!)



- Byg
- w
- Ag MM
- C10
- C12
- C13
- C17
- C19
- C20
- C10
- C12
- C13
- C17
- C19
- C20
- Asc MM
- w
- Byg

Take ✓ 12 µl NEB buffer 4 X10
✓ 105 µl ddw
✓ 3 µl (30v) Asc I lot 10 (10v/µl)

19 µl + 1 µl of J43 minis (C10) (C12) (C13) (C17) (C19) (C20)
37° 2:20 pm to 4:40

~5 µl to 11 µl
as Post Asc
(C10) - (C20)

Each plus 5 µl
4 X R1 4 X enz
back to 37° 4:52 to 6:30

(4 X R1 4 X enz)
✓ 12 µl 10 X RI salts
✓ 12 µl ddw
✓ 3 µl (30v) Not I lot 48
✓ 3 µl (30v) Bgl 2 lot 35
30 µl

Post
Ag, Bgl, Not
Cold

to cold

3/10 Sat. July 17th

1.294 x 1
RNAse
3/10

Recheck with better conditions

(C20) (T19)

(2) Take ✓ 8 µl 10 X NEB buffer 4
✓ 70 µl ddw
✓ 2 µl (20v) Asc I lot 10 (10v/µl)

2 µl J43 (C20) 37° 10:05 am 2 µl J41 (T19)
to 11:43 + 3.5 µl 8M NH₄ Ac

8+8 AB N 8+8
20 Asc 19 Asc

Heated 80° 15 min
+ 100 µl ETOM .F.C.D. ✓
black into 20 µl 1 X RI cells 4 µl 10 X
37° 1:25 pm to 4:10 pm 2 µl Not I ✓ 32 µl ddw
2 µl Bgl II
Undiluted
lot 38

DrdII MneI

541 TAATGTTTCTGGTCTCTTGGCTCTTAATATGCTCTCATCTCAGCAGAGTCGGAAGAAG
 ATACAAAGACCAAGAGAGCGGAGAAATTATACGAGAGTAGAGTCGTCTCAGCCTTCTTC 600

Y V F W F S C L L I W L S S Q Q S R K K -
 M F S G S L A S * Y G S H L S R V G R R -
 C F L V L L P L N M A L I S A E S E E G -

DrdII Tth11111 RcaI

601 GCGTGAACCAACAGACTTGGGAGTCACTAGAACACAGATCATGCGGCTCAATATGAAT
 CGCACTTGGTTGTCTGAACCTCAGTGATCTTGTCTAGTACTGCGGAGTTATACTTA 660

A * T K Q T W E S L E T R S * R L N M N -
 R E P N R L G S H * K Q D H D G S I * M -
 V N Q T D L G V T R N K I M T A Q Y E C -

EcoO109I Psp5II Tth11111 EcoO109I HaeI Psp5II Sse8647I

661 GTTACCAGAAGATCATGCGAGGCCCATTCACAAGCAGAACTTACTGCAATAGGA
 CAATGGTCTTCTAGTACGTCCTGGGTAAGTTGTCTTCTCGGAATGACGTTATCTCT 720

V T R R S C R T P F N K Q R A F T A G -
 L P E D H A G P H S R R R P L L O G D -
 Y Q K I M Q D P I Q Q A E G L Y C N A T -

My Exon 2

My Exon 3 = Rat Exon 3

721 CCGGGGCGGATGGCTATGCTGGAATGACGTTGCAGCAGGAGCGAATCAATGCGAGTACT
 GGACCTGCTCACCAGATACGACCTTACTGCAACGTCGTCCTGCTTAGTTCGTCATGA 780

P G T D G Y A G M T L Q Q G R N Q C S T -
 L G R M A M L E * R C G R D G I N A V Y -
 C W N D V A A G T S M Q Y

J49 Mouse Exon 3 Blue

AG BglII BstVI

781 GGCCTGACTATTTTCAGGATTGTATCCTTCAGAGGTTACAGATCTGTGACCAAG
 CGGGACTGATAAAGTCTCTAACTAGGAAGTCTCTCAATGTTCTAGACACTGGTTC

A L T I F R I L I L O R R L O R S V T K -
 P * L F S G F * S F R E G Y K D L * P R -
 P D Y F Q D F D P S E K V T K I C D Q D -

BsaWI BspEI Exon 4 DraIII

841 ATGGCACTGGTTTCGGCATCGGATAGTAATAGAACATGGACCACTACACCTGTGTA
 TACCTGTGACCAAGCGTAGGCTATCATTATCTGTACCTGGTTGATGTGGGACACAT 900

M D T G F G I R I V I E H C P T T P C V -
 W T L V S A S G * * * N M D Q L H P V * -
 J49 Mouse Exon 4 Red

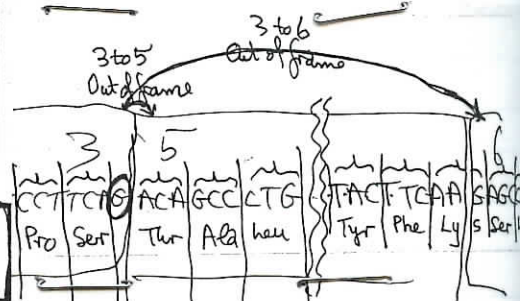
901 ATAAAGCAGCATGAGAAAGTGAAGCAGCCCTGAATCTGTTCTACCTAATATAATTG
 TATTGTCGTGCTATCTTTCACTTCTGTCGGGACTTAGACAAGATGGATTGATATTAAC 960

I T A R M R K * R Q P * I C S T * I * L -
 * O H A * E S E D S F E S V L P N Y N W -
 N S T H E K V K T A L N L F Y L T I I G -

BclI HinfI My Exon 5

961 GACATGGATTATCTATGCACTCTGATCATCTCTCATCATATTTTCTACTCAAG
 CTGTACCTAATAGATAACGTAGAGACTAGTAGAGAGAGTAGTATAAAAAATGAAGTTC

D M D Y L L L H L * S S L S S Y F F T S R -
 T W I I Y C I S D H L S H H I F L L Q E -
 H G L S I A S L I I S L I I F F Y F K S -



Shifting frames for
3 to 5 and 3 to 6

132. seq

CRAMUS

Exon 3

5151. CNACCCATTAAACAAATCTCTATAATGATGTAAGAAATAGAAA 5200

1 ... GGCCGGATGGCTATGCTGGAATGACGTTGCAGCAGGGACGGAATCAA 47

5201 AGAAGAAAGAAAGCAAACTAATGATGTTAAATACATTATATAGATCA 5250

48 TGCACTACTGCCCTGACTATTTCAGGATTTTGATCTTCACATGAGAC 97

5251 AAGGATCTTTTAAATTTCCATATCAGATGCTCTGCGCTCAAAATTT 5300

98 CAGAAATTT Part of Exon 3 AATTACAGATCTGTCTGAGCCTCAAAATTT 136

5301 G. GAAAGAAATAGCAAAACCTCAATAGGTAAGAAAGCCTTATCAGGTA 5349

137 GTGAAAGAAATAGCAAAACCTCAATAGGTAAGAAAGCCTTATCAGGTA 186

5600 TTTTAAAGTAGAATCTATTAATTTGAATTTTATCTAATATATATGAGA 5649

437 TTTTAAAGTAGAATCTATTAATTTGAATTTTATCTAATATATATGAGA 486

5650 TTTTATAGATCAGGGGTCATTCCATTTTCTTACCACACAATTA 5699

487 TTTTATAGATCAGGGGTCATTCCATTTTCTTACCACACAATTA 536

5700 ACAGCCCTGAATCTGTTCTACCTAATATAATTTGGACATGAGGATATTA 5749

537 ACAGCCCTGAATCTGTTCTACCTAATATAATTTGGACATGAGGATATTA 586

5750 TTGCATCTCTG. 5760

587 TTGCATCTCTGATCGCGCGCCATAACTTGTATAATGTATGCTATACGA 636

Exon 5

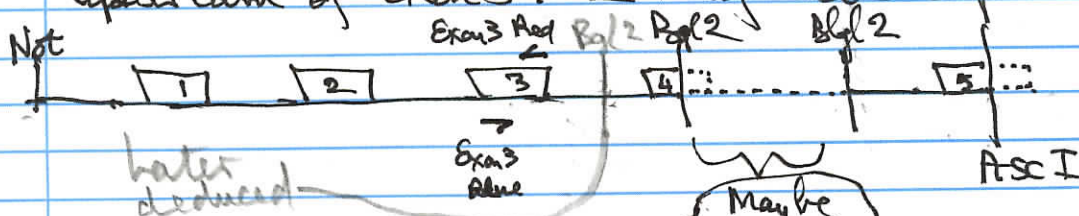
Conclusion from 132. seq
 is that a b12 fragment was
 lost covering exon 4. Splice
 will probably occur from 3 to 5.

give no trouble. But if it
 occurs from 3 to 6 it will
 also be out of frame (See above)

Sat - July 17th

Final sequencing checked on T19

Netblast search of CRLRMUS SEQ against Genbank showed CRLR Rat (L27487.gb-ro) is no longer the best match. There are now two mouse CRLR mRNA sequences (ab015595.gb-new and af146525.gb-ro) that are better ~100% identity to my exon 1, exon 2 & exon 5 sequences. Can design new primers to pick up exons 3 and 4 in T19 cDNA. A possible problem is that the sequence in Genbank has a Bgl2 site in exon 4 that is not equivalent to my Bgl2 site upstream of exon 5. I may have a problem:-

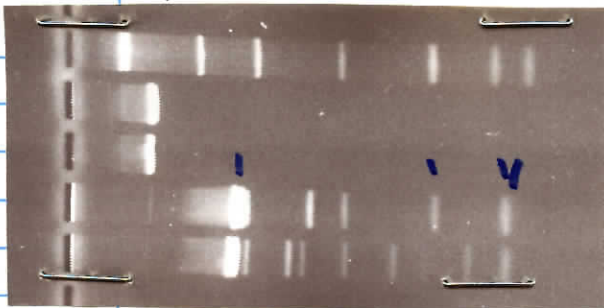


essential to see that area maybe missing sh'd be visible in 129 seq when finished. Design the Exon3 Blue to give +ve even if Bgl2 is as published. Exon 4 Red will fail if that sequence is missing. Not very critical, but it may affect exon skipping

Water	Template	Oligo #	23153	5'	Oligo Name: J'49 Exon 3 Blue	3'
Seq 132 14	J'49 Exon 3 Blue/100 4pp	23153	DATE made: 20	5'	CCT TATA CTGG CAIA TAG	3'
Seq 133 14	J'49 Exon 3 Red/100 4pp	23184	DATE made: 19	5'	TAG TCA GGG CAAG TAC	3'
Seq 134 14	J'49 Exon 4 Blue/100 4pp	23185	DATE made: 20	5'	AGA GAA GGT TAC AIA	3'
Seq 135 14	J'49 Exon 4 Red/100 4pp	23186	DATE made: 20	5'	ATG CIG GCT GTT ATT	3'

Perfect match to 132 seq

14.8 kb
13.5 kb



2.5 kb
2.5 kb

Conclusion: Pretty certain that
Bgl2 is incomplete.

PK/ON 6
1/10
3+
3+
2+
1

Use ~~(C19)~~ J'43

~~4pl NE Buffer 3 x 10~~

~~pelaw
pelaw~~

1 pe(100) ~~100~~ 200 (u/pe)

~~Old tube~~

New tube

4 μ l NE Buffer 3×10

4. $10 \times 10 \times 10$ BSA

५१५५५

~~pe C195'43~~

~~20
↓
lpe(v) Not
hat
(v) pe)~~

del + a

20
pec ✓) Not 1
hot
(v/pl)

New note

carriers from J47

Sat. July 17th

~~6:30 am~~
~~9:30 am~~
8:19 H+K
12:15 W+N
R.N.A.C.T.
13:10 T

A/G

3+12
3+12
2+14
T+14

Dyrr
Dyrr
T
~~T~~
~~T~~
T19
C20
W
Dyrr

C19 & C20 AscI, BglII + NotI digest 1/4th diluted 2 μ l + 14 μ l (i.e. 6 $\times$ 8).

~~100~~ T19 } ASC (already diluted once) from J47
~~100~~ C20 }

T19 } I₄ 7 Asc, Bgl, Not default
C20 }

W
Dyrt

• logic of paired enzymes.

Nothing odd - Forget Egl 2 sites
in Dup Del at 2150, 2160 & 240

Sun. July 18thPaired Digests! (Rough)

① 24:20^{pm}
 4:55^{pm}
 8:10^{pm} 10^{pm} 3/10
 Bgl II
 W
 ANA

AGMM
 J53 Bgl Not T19
 AcRMM
 W
 Bgl

Asc I + Not I, Asc I Bgl II, Bgl II + Not I

Take 80 μ l 10 $\times$ R1 salts70 μ l ddH₂O2 μ l T19(200) 2 μ l Bgl II Lot 36(200) 2 μ l Not I Lot 48

9:33^{am}
 10:03^{am}
 1.2 μ l H₂O
 10 μ l 3/10
 RNAse

Bgl
W

②

AcRMM

J53 Bgl Not T19

" " " C20

J53 Bgl Not Asc T19

" " " C20

AcRMM

W

Bgl

Take 80 μ l 10 $\times$ R1 salts70 μ l ddH₂O2 μ l C20(200) 2 μ l Bgl II Lot 36(200) 2 μ l Not I Lot 4840 μ l20 μ l+ 1 μ l Asc I (100) Lot 1037° 5:50^{pm} - 9^{am}37° 5:50^{pm} - 9^{am}Mon July 19th

To confirm control check for primers

Primer

Template

Submitted
 July 19
 1993

Seq. (3)	Water 14 μ l	G ^{III} TK4731/100 5 μ l	J'43 mini 1 μ l
			C20

Confirms that C20 comes in from 3' end of total sequence
 & overlaps with EcoRV end of targeting. CRLRMUS
 sequence is very well confirmed over whole of control extension.
 Data are over page on J54.

37° 1:40^{pm} - 4^{pm}

New tube
 J53
 Bgl Not
 T19

20 μ l + 1 μ l Asc I (100) Lot 10
 (100 μ l)

37° 4:20^{pm}

J53
 Bgl Not
 Asc
 T19

J53
 Bgl Not
 C20

J53
 Bgl Not
 Asc

cr1rms.seq x 131.seq July 22, 1999 10:04 ..

7531 GGAATTTAACTGNATTCTCTACAAGAGTAGCAAATTCCTTAGACACTG 7580
 899 NGGAATTAAC..GGTNTCTCAAAGAG.....AATTTTGTAGNCA... 863
 7581 ATGCATCTCTCTAGCCCCAGTTTTCACATATATTTAGTTTATTATTGC 7630
 862 TNGATCTC..CTAGCCCCAGTTTTCACAAATAGTTTA.....TAAGC 821
 7631 AAACCTTTATATTTTTCAAAGCCTGCAATTCCTTGGTCAAATATATGT 7680
 820 AACCC...TTTATNTTNCANCCNTNC..ATTTNTTGNCAANA...GT 779
 7681 GTTTTGTGCTTCTCTTGAAGTAGCAATTCAAAGCTCTCTAAAGCA 7730
 778 GTTTTGG...GCTTCTGAA..TAGAGCAA..TCAAGCTCTCTAAAGCA 735
 7731 ATATTCTCAGAATGAATTCATAGCATCCAGCTCTTCCAAACCTAAGTAG 7780
 734 ATATTCTCAGAATGAATTCATAGCATCCAGCTCTTCCAAACCTTAAAGAG 685
 7781 ATTTCTTACNAACTAACCCATCTTACCCGGTGATATCCAGAGGTA 7830
 684 ATTTCTTA..CAAACTAACCCATCTTACCCGGTGATATCCCA..AGCTCA 637
 7831 GTTTCATTTCAGAAATTTAGCAATTTAGAGATCCATTCTGCCCTTT 7880
 636 GTGGCATTTCAGAAATTTAGCAATTTAGAGATCCATTCTGCCCTTT 587
 7881 CATTTCTCCTTATAGGCTTTTCATATCTCTCAATATCCAGTTTAACT 7930

exon3.seq x af146525.gb_new August 2, 1999 15:31 ..

869 AGCCCTTACTGCAATAGGACCTGGCCGGATGGCTATGCTGGAATGACG 918
 374 AGCCCTTACTGCAATAGGACCTGGGACGGATGGCTATGCTGGAATGACG 423
 919 TTGCAGCAGGGACGGAATCAATGCAGTACTGCCCTGACTATTTTCAAGAT 968
 424 TTGCAGCAGGGACGGAATCAATGCAGTACTGCCCTGACTATTTTCAAGAT 473
 969 TTTGATCCTTCAG 981 (GT)
 474 TTTGATCCTTCAG 486 (GT)
 8181 TGCATCAAATGCAGAGCTTATTTGTTATTTCTGCTTCCAAACATTAGAG 8230
 286 TGCATCAAATGCAGAGCTTATTTGTTATTTCTGCTTCCAAACATTAGAG 237
 8231 TACTTCTTTTCTGCACCTTACATGAACATGAAAGTGTAAATGAA 8280
 236 TACTTCTTTTCTGCACCTTACATGAACATGAAAGTGTAAATGAA 187
 8281 TCTTGCTATCATTTTCTGCTCCCTAGCCTGGTTAAATGCTTCATAT 8330
 186 TCTTGCTATCATTTTCTGCTCCCTAGCCTGGTTAAATGCTTCATAT 137
 8331 CTGGGAATTTTACTGGATCC 8350 (C20)
 136 CTGGGAATTTTACTGGATCC 117

Agood.
 sequencing
 primer
 } I 151 Red } J 55 Red
 but not very
 discriminating on PCR

Looks ok on PCR
 } I 150 Red

af146525.gb_new x 132.seq July 23, 1999 10:40 ..

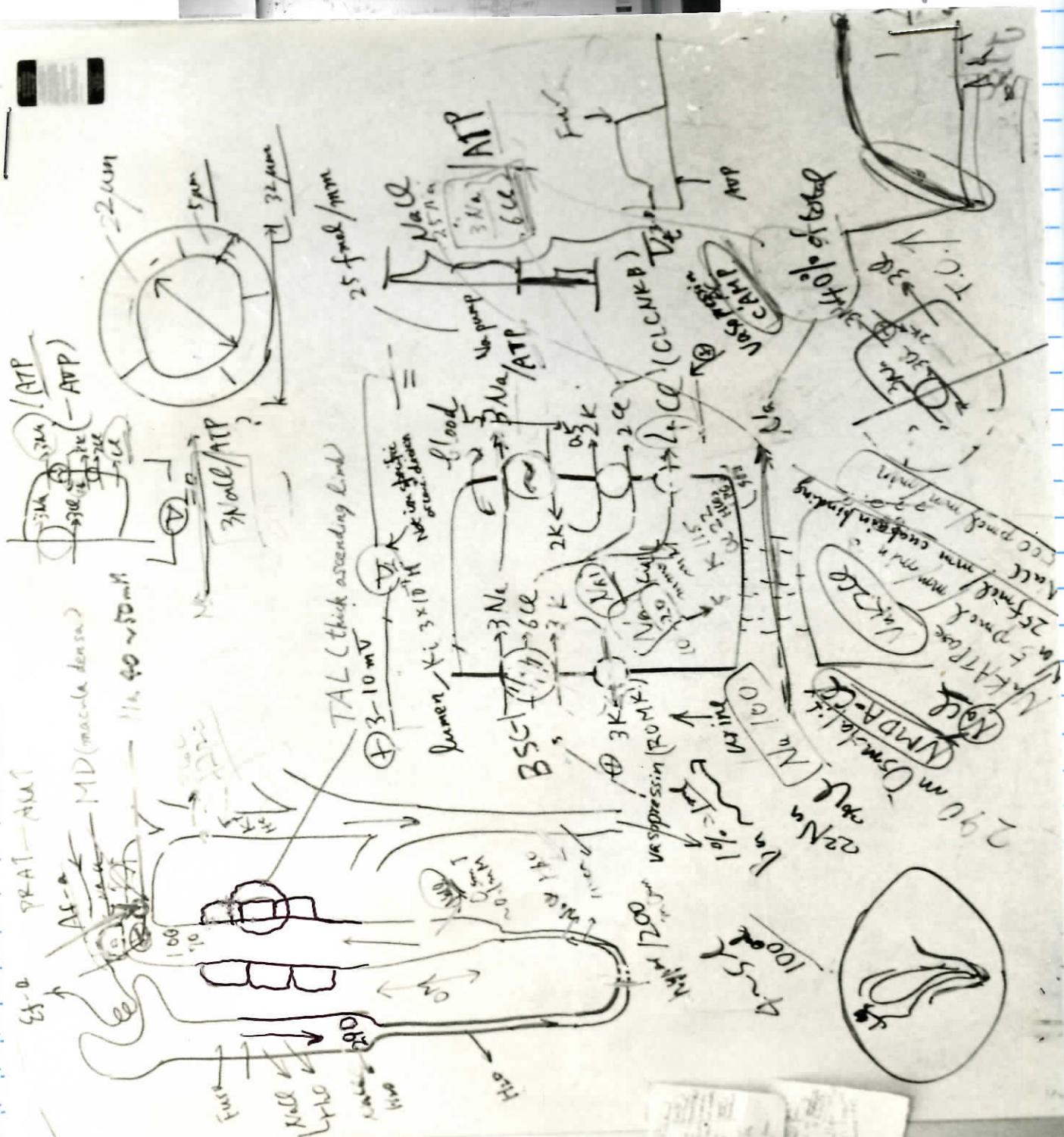
350 GCAGGACCCCATCAACAAGCAGAGGCCCTTACTGCAATAGGACCTGG 399
 1 Exon 3 399 2
 400 ACGGATGGCTATGCTGGAATGACGTTGCAGCAGGGAAGGAATCAATGCAG 449
 3 CCGGATGGCTATGCTGGAATGACGTTGCAGCAGGGAAGGAATCAATGCAG 52
 450 TACTGCCCTGACTATTTTCAAGATTTTATCCTTCAG...AGAGAAGGT 493
 53 TACTGCCCTGACTATTTTCAAGATTTTATCCTTCAG...AGAGAAGGT 102
 494 TACAAAGATCTGTGACCAAGATGGACACTGGTTTCGGCATCCGGATAGTA 543
 102 TACAAAGATCTGTGACCAAGATGGACACTGGTTTCGGCATCCGGATAGTA 152

J49
 Exon 3
 Red

Good
 sequences
 Submitted
 July 26

Water Primer Template

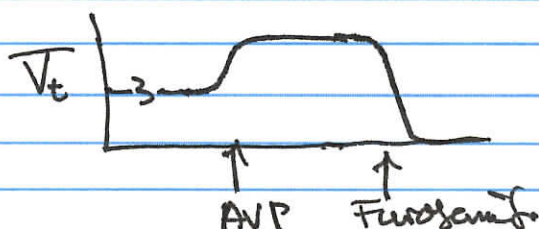
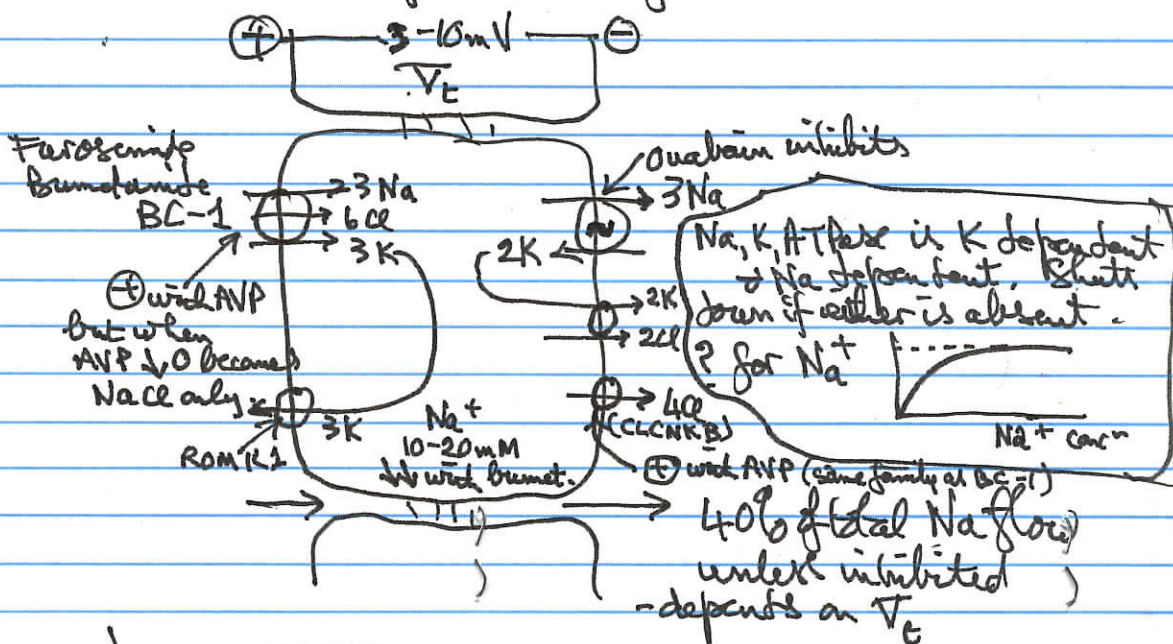
Seq 136	14pl	J 55 Red	1pl	(C20)
		5pl 100	J 43	mem
Seq 137	14pl	J 55 Red	1pl	(C20)
		5pl 100	J 43	mem



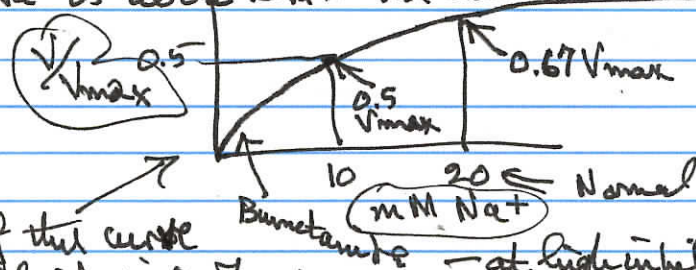
Fri. July 23rd

Computer modelling of TAh cell [see also J'69]

Meeting this am. with Nobuyuki Takashi
 & this afternoon with N.T. and Arlen Anderson
 led to several useful ideas for model.



looks like we should assume that Na,K,ATPase is on an insensitive part of Na curve ~~also~~ in wt & hetero, but gets very sensitive with homo. A likely 0.5 for Na is about 10 mM but intracellular may be 20 mM.



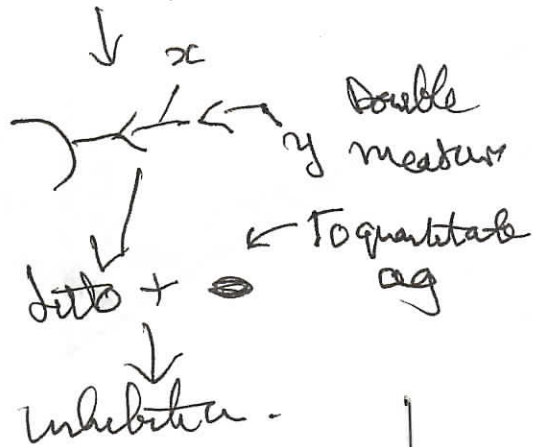
So a 25% ↓ in heterozygote when BC-1 changes to 50%

The shape of this curve is probably v. important for what happens in life & how the model behaves

at high inhibition the Na⁺ ↓ & pump stops.

(S)BIA

Single bead immunoassay



multi
 micro
 nano
 pico
 femto
 atto

Sunday Aug 1st

Same thoughts on surrogate markers for hypertensive hypertrophy

Meetings on design of RFA for above have suggested the value of several peptide hormones as surrogates using plasma concentrations. The difficulty is the low conc. of many & the limited availability of plasma to the laboratory assays. Sat. July 31st with Bryan Ballou suggests that the limiting factor is getting enough signal into a small enough volume for assay. For example:

milli - 10^{-3}
micro - 10^{-6}
nano - 10^{-9}
pico - 10^{-12}
femto - 10^{-15}
atto - 10^{-18}

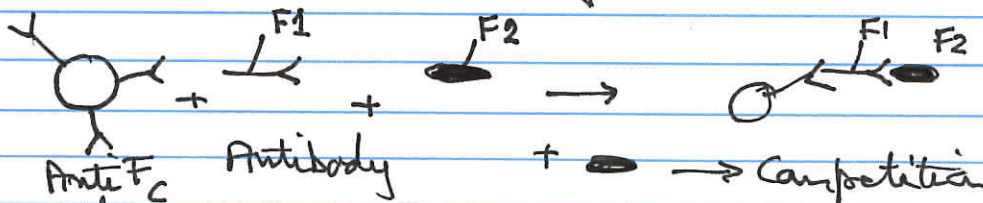
$$\begin{aligned} \text{Ang II is } & \sim 23 \text{ pg/ml in wt.} = \frac{23 \text{ pmoles}}{10^6 \text{ femtomoles}} / \mu\text{l} \\ & \text{kin} \rightarrow \frac{23 \times 10^3}{1046 \times 10^3} \text{ pmoles} / \mu\text{l} = 22 \text{ femto moles} / \mu\text{l} \end{aligned}$$

Detection limit of cyanine dye fluorescence with high quality Wallac instrument is $\sim 10 \text{ fmoles} / \text{well}$
 - so $10 \mu\text{l}$ w'd be at limit of even approx conc. measurement
 With Time Resolved Fluorometry sensitivity can be $\uparrow \sim 1000 \times$
 to $\sim 10 \text{ attomoles} / \text{well}$, but then requires labeling with a lanthanide chelate.
 Dana Fowkes uses 2 Samarium & Europium to get calibration.

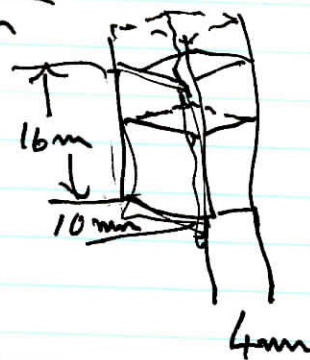
This is range of affinities.

$$22 \text{ femtomoles} / \mu\text{l} \rightarrow \approx 22 \text{ nanomolar}$$

Will try all of this without concentrating the peptide - Kim starts with $600 \mu\text{l}$ of plasma but will up with $3 \times 200 \mu\text{l}$ samples for RIA ~~assays~~ dried down.
~~isn't detecting, somewhat by this method~~



Sun Aug 8th B.B. + Alantignier will investigate this idea.

	S.H	N.S.T	①
V	3.00 V	90 V	
V/cm	750 V/cm	600 V/cm	
capacitance	250 pF	60 pF	
length	4 mm	1.5 mm	
area	160 mm ²	17 mm ²	
Resistance			
volume	650 μl (mm ³)	25 μl	
	$R \propto \frac{\text{Gap length}}{\text{Area}}$		
	$\frac{R_{S.H.}}{R_{N.S.T.}} = \frac{4}{160} \times \frac{17}{1.5} = 0.28$		
	$\frac{RC_{S.H.}}{RC_{NST}} = 0.28 \times \frac{250}{60} = 1.17$		

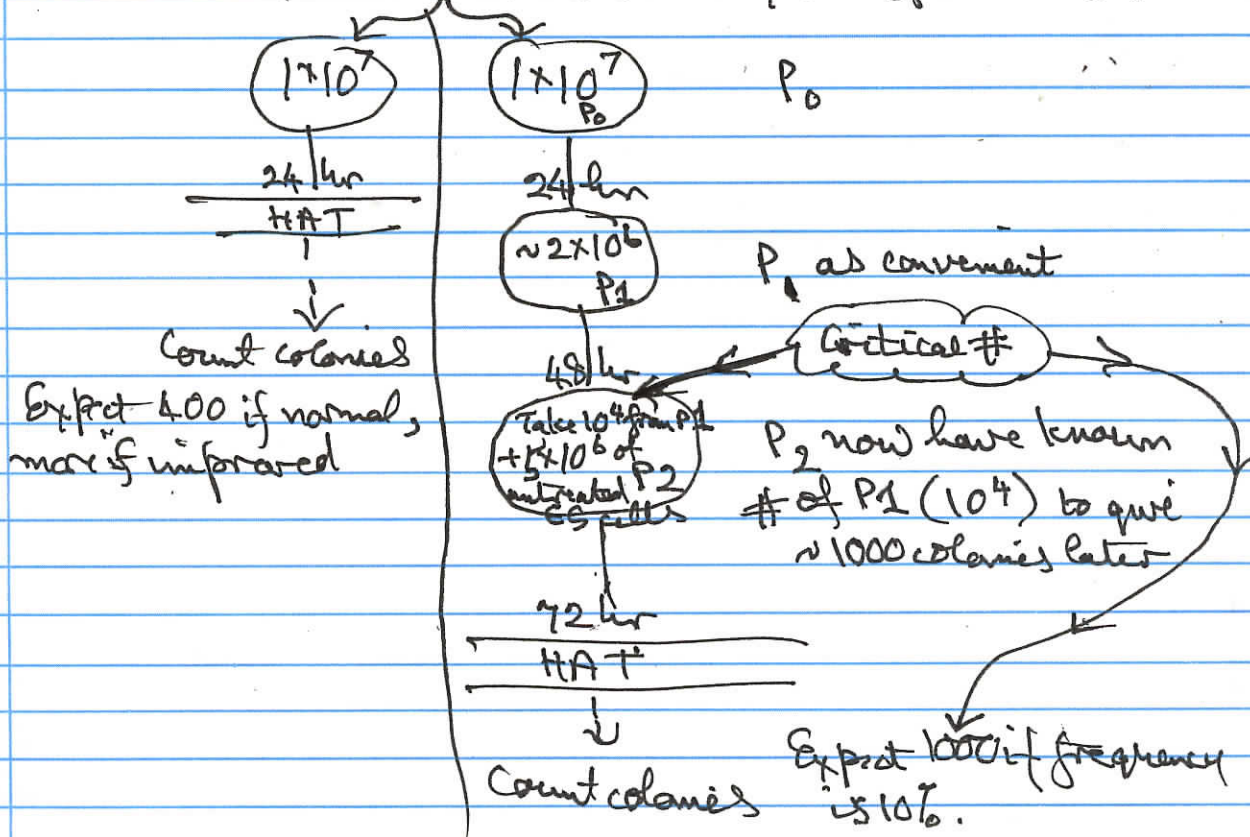
time constants are same
but our voltage is ~25%
? reduce to 200 V.

Monday Aug. 9th

Targeting Efficiency

Reviewing literature for Hb grant renewal reveals a paper using E4TG2a & mp8, claiming that gene targeting is really closer to 10^{-1} absolute than the usual estimate of 10^{-5} . Careful review, with Seigo & Nobuyo, shows very few differences from our regular procedure except in when selection is imposed. Thus V/cm are similar, our DNA conc. is 3x lower, RC time (estimated from areas of cells and capacitors) are close. E.p. is at 4° and cells are allowed to sit before plating. Biggest difference is that selection is imposed at > 60 h with HAT & cell platings are at higher density. Tried to get a protocol that allows comparison:-

↓ Voltage from 300V to 240V, reduce capacitance from 250pF to 200pF, increase DNA from 10µg (1.6nM) to 30µg (5nM),
↓ E.p. temp. to 4° from rt., pre & post incubate for 20 min vs. ~10 min. & ~2 min. Plate at 1×10^7 / P100 (from 5×10^6).



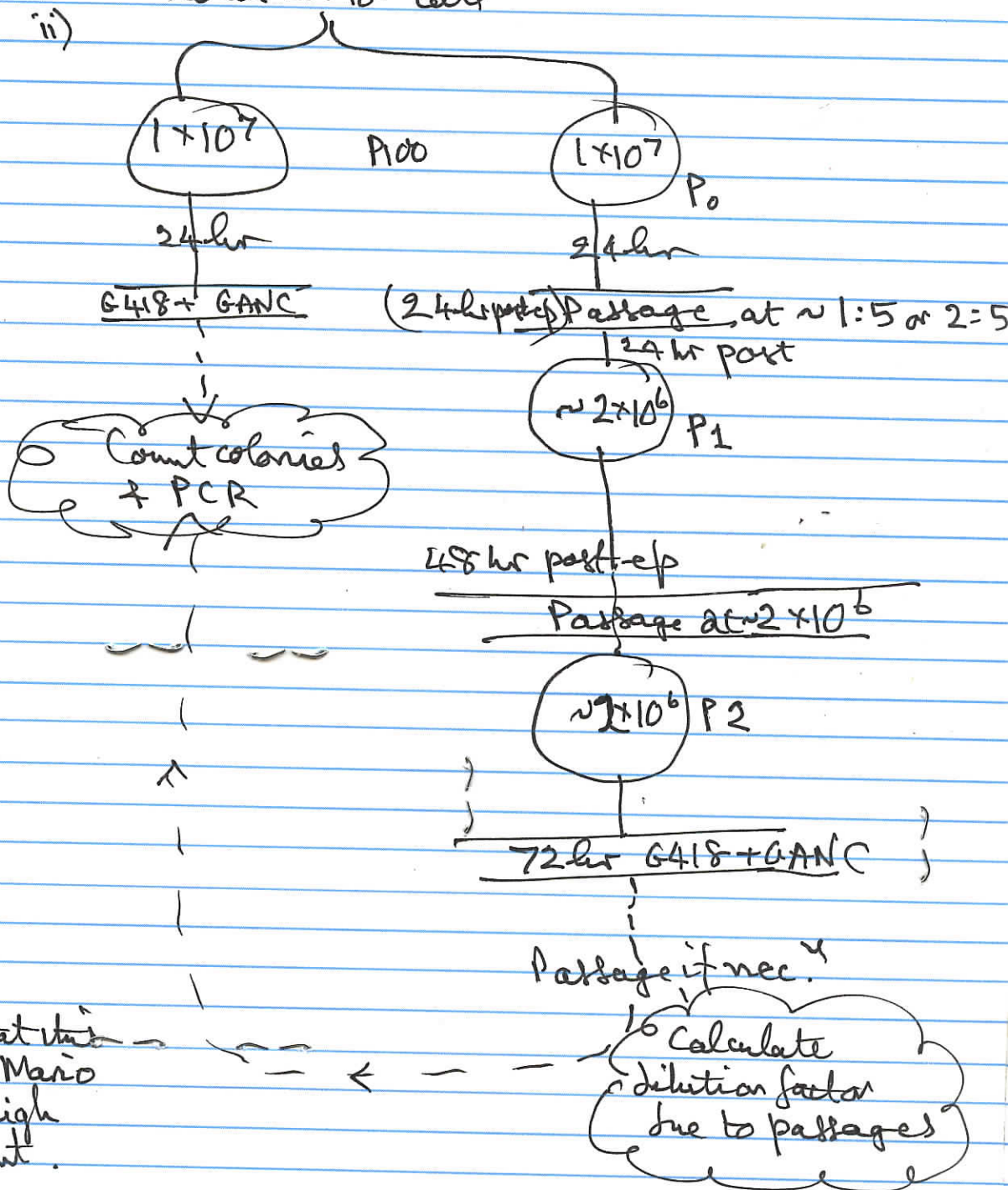
Mon. Aug 9th

Targeting Efficiency (contd.)

Can adapt the NST protocol to G418 & GANC? —

i) Ep at 4° with pre & post incubation of 5 nM DNA with 2×10^7 cells

ii)



It could be that this accounts for Mario Capecchi's high enrichment.

Please find enclosed pJC13-1.

Best Luck,

Adam C. Bell, Ph.D.
Research Associate
National Institutes of Health
Building 5, Room 218
9000 Rockville Pike
Bethesda, MD 20874-0540
phone (301) 496-1898
fax (301) 496-0201
email adumb@bldg5.niddk.nih.gov

*The construction of pJC13-1 is described in Cell 74, pp.505-514.

•The base plasmid is pGEM4Z.

*The sequence of the 1.2Kb chicken β -globin insulator can be found at Genbank #U78775.

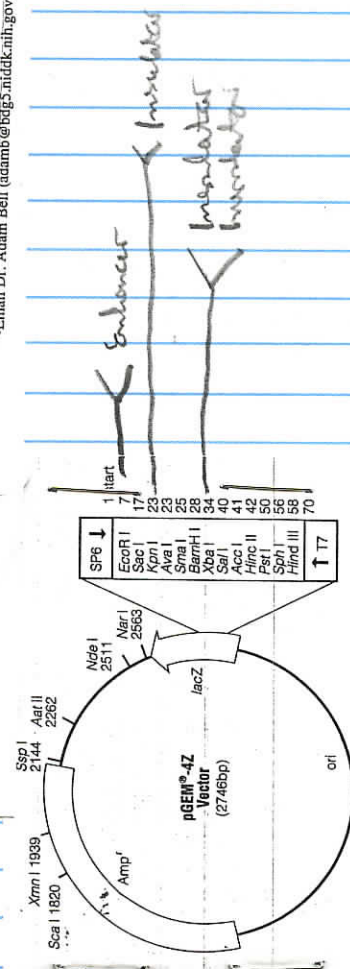
The arrows indicate the orientations of the cloned fragments relative to their native orientations or those indicated in Genbank.

To obtain a single ~2.4Kb fragment containing two copies of the 1.2kb insulator, digest pC13-1 with BamHI and EcoRI or BamHI and SalI (double digestions in both cases). Be careful to avoid the closely migrating ~7.7Kb BamHI fragment.

All of the restriction sites shown are found only where indicated. Thus, the enhancer can be removed by digestion with EcoRI and the γ -neo reporter can be removed by digestion with BamHI.

Our experience is that cloning large (>10kb) fragments between double insulators can be difficult, if you have trouble cloning, try STBL2 cells from Life Technologies. In general, these constructs can be difficult to obtain, but once isolated they are stable upon re-growth in *E. coli*.

Email Dr. Adam Bell (adamb@bdg5.niddk.nih.gov) if you have additional questions.



Fri. Aug 22nd

"Insulators"

To test the proliferative advantage concept
in the β globin region I propose to surround
the transgene with the elements described
by Gary Felsenfeld et al. (see, for example, Adam C. Ball
et al. Cell 95: 387-396 Aug 6, 1999 & Howe et al.,
J. Human Genet. 44: 152-162, 1999). Received the
plasmids p JC13-1 today. S.H. will grow up.

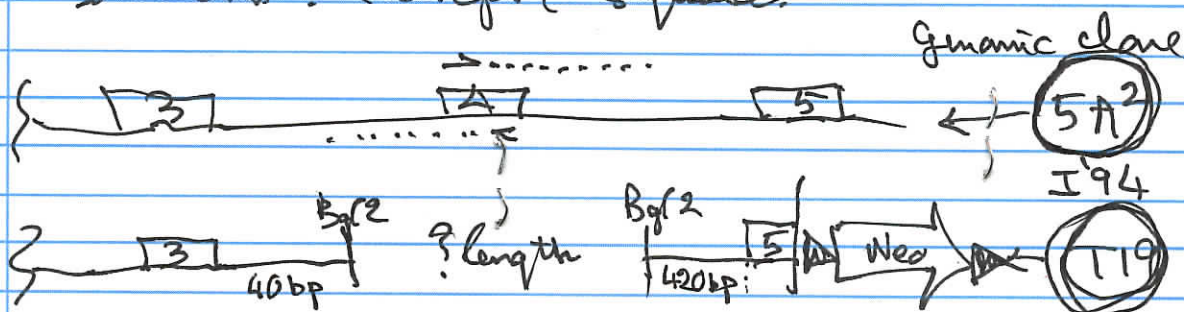
↓ as J'65 SHc106
1.517 μ g/ μ l

I will call it J'65 JC13-1

Tues. Aug 31st

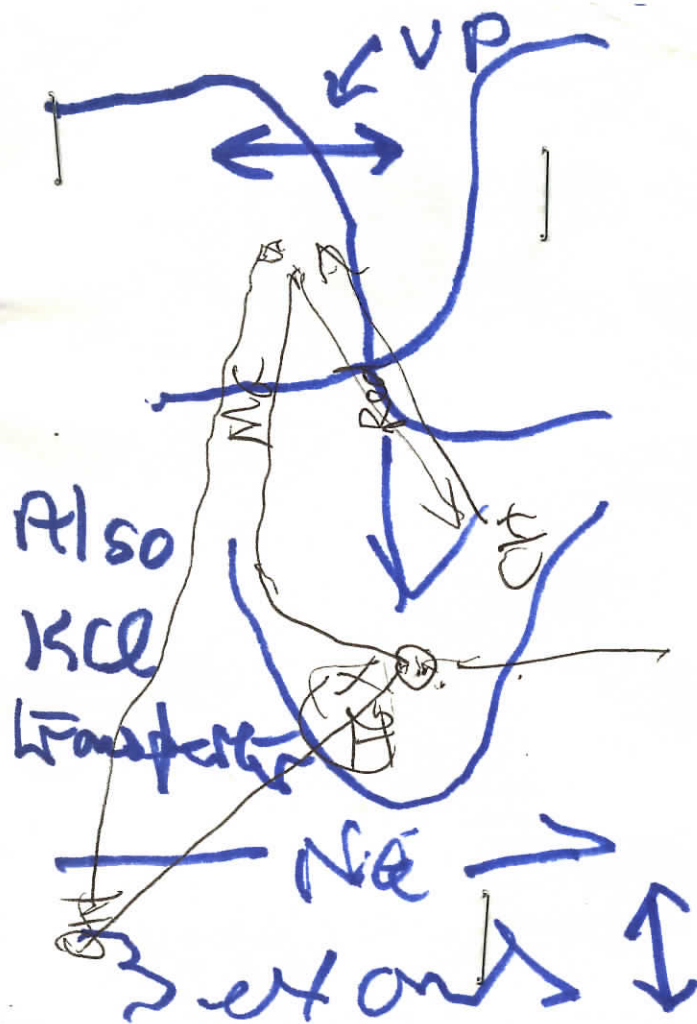
Check on Exon 4 deletion in Targeting Vector for CRLR

Need Bgl2 deletion length for predicting Southern. Therefore sequence:-



Sequenced Aug 31

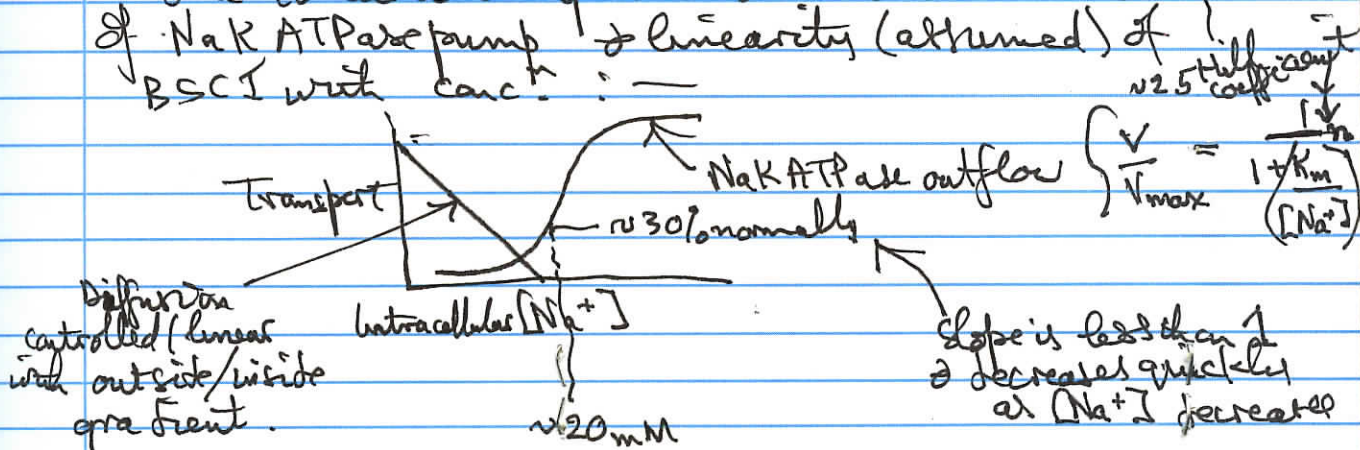
	Ddw	Template	Ames
Seq 138	101P ✓	5pL 5A2 ✓ I'94 1/10	5'49 Exon 4 5pL ✓ 100
Seq 139	101P ✓	5pL 5A2 ✓ I'94 1/10	5'49 Exon 4 5pL Red ✓ 100



Sunday Sep. 19th

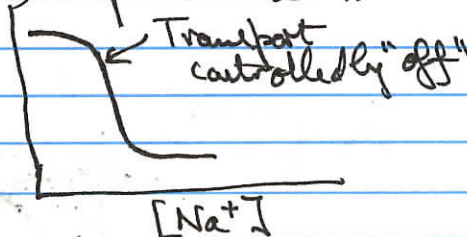
Extension of TAL computer modeling [See 157]

During a meeting on Fri. Sep. 17th, Arlie Andersen presented latest simulation! He can get the system to come to a steady state, but when BSC1 (NaK2Cl) transporter is reduced to half the net transport of NaCl decreases. The decrease is not 50% so some effect as predicted is occurring. The problem was pin-pointed as due to the assumption on cell sensitivity of NaK ATPase pump & linearity (assumed) of BSC1 with conc. :-

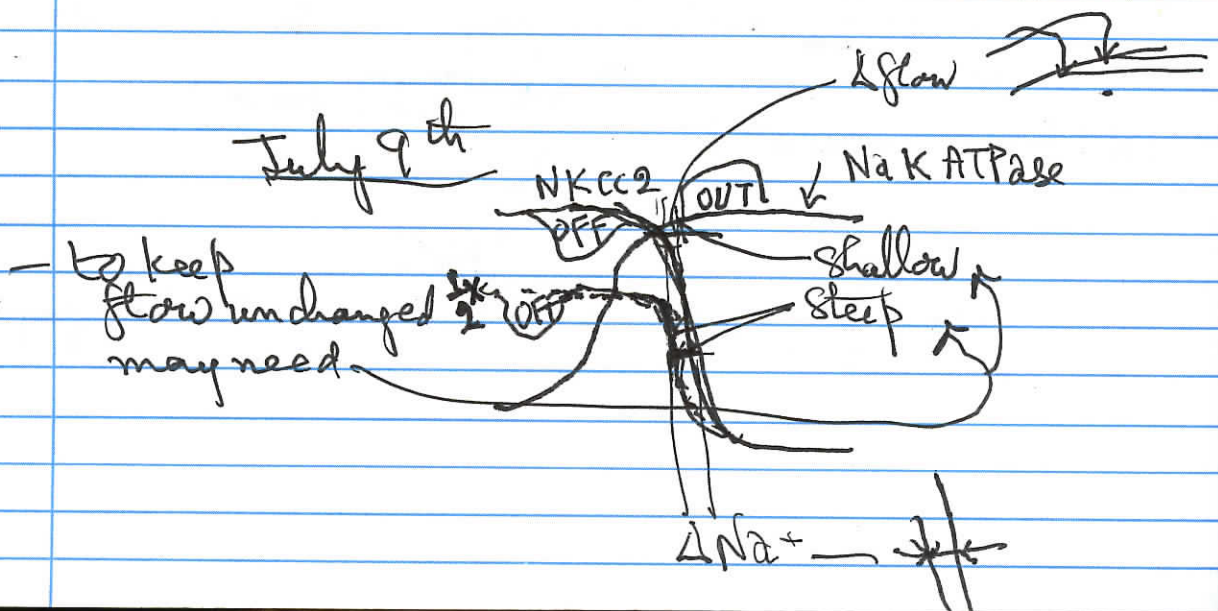
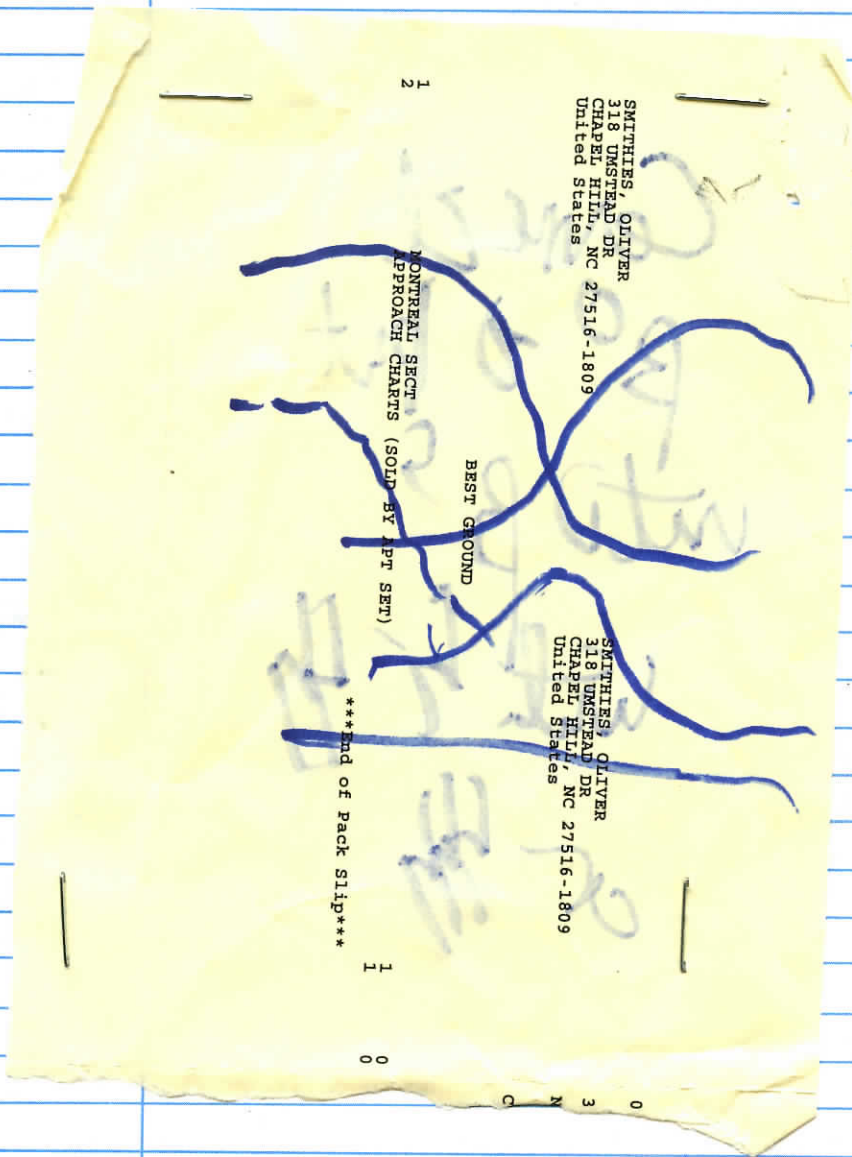


Possible solⁿ. @ w'd require BSC1 transport to respond to outside/inside gradient more than linearly. O.S. solution - assume that NaK2Cl cotransporter has an off (inside) response that has an inverse $[Na^+]$ sensitivity.

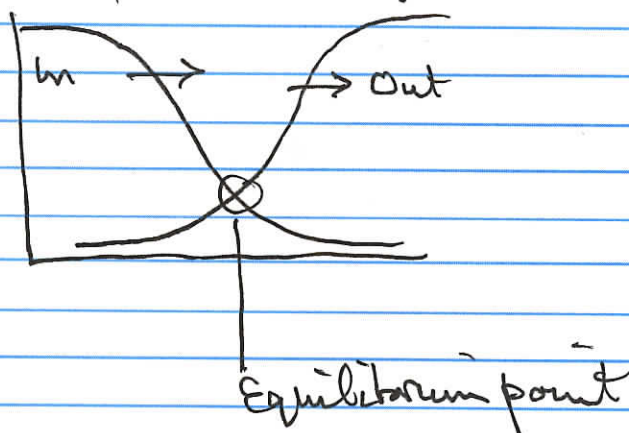
(N.T. "...you're a genius...")



The net result will be a self-stabilizing intracellular $[Na^+]$:-



TAL modeling (contd.)



A slight shift in either direction will act to restore $[Na^+]$ inside the cell.

Corollary 1

The total flow ~~can~~ will be controlled by AVP action on BSC-1 — not by NaKATPase, which will just follow along. This suggests that the ~~the~~ KCl flow may be similarly controlled by ~~the~~ AVP acting on other channels.

Corollary 2

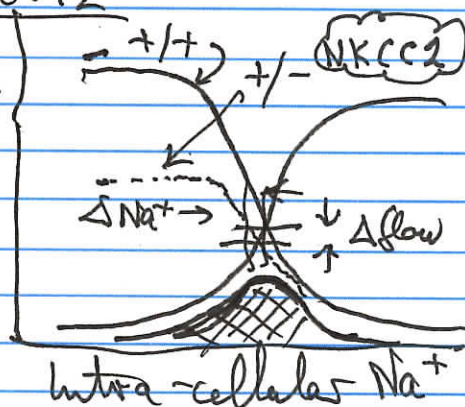
The BSC-1 cotransporter has to operate with different extra-cellular (urine side) concentration of $[Na^+]$ or $[K^+]$. Hypothesis: the 3 splicing isoforms of BSC-1 gene have evolved to adjust the sensitivity of the cotransporter to external $[Na^+]$ to keep the flow under control over whole length of TAL.

Tues. Oct. 12th

Update

The curve is upside down!

Flow



Dr. Oliver Smithies
Excellence Professor of Pathology
Dept. of Pathology and Laboratory Medicine
CB # 7525
701 Brinkhous-Bullitt Building
Chapel Hill, NC 27599-7525

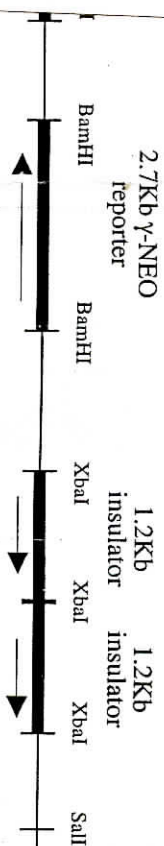
Please find enclosed pJC13-1.

Best Luck,

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email adamc@bldg5.niddk.nih.gov

JC13-1



Cell 74, pp.505-514.

insulator can be found at Genbank #U78775.

oned fragments relative to their native orientations or those

ons of the insulators and reporters is unlikely to influence stability.

ng two copies of the 1.2kb insulator, digest pJC13-1 with BamHI
ions in both cases). Be careful to avoid the closely migrating

only where indicated. Thus, the enhancer can be removed
er can be removed by digestion with BamHI.

•Our experience is that cloning large (>10kb) fragments between double insulators can be difficult.
If you have trouble cloning, try STBL2 cells from Life Technologies. In general, these constructs can be
difficult to obtain, but once isolated they are stable upon re-growth in *E. coli*.

•Email Dr. Adam Bell (adamc@bldg5.niddk.nih.gov) if you have additional questions.

~ Sep. 25th

"Insulators" (redundant entry)

Following reading of Bell, West & Felkenfeld, Cell
98 387-396 on use of insulators for transgene
isolation talked to Gary & got encouragement
for use with EPR. The requested contract
arrived & has been grown up by S.H. as J'65

I will call it

J'65
SC13-1

SHC106
1.517 pg / μ l

Fri. Oct. 29th

Grant completed - nearly 3mo., & sent off!!

Antibody staining of RBC

- before deadline!

~20 μ l of heparinized blood from J.L.
Washed 3x with ~1ml PBS at R.T.
Resuspended & added 1ml 4% formaldehyde
(37% formalin 1 vol. + 8 vols. PBS)

0 min
2 min
0 min
4 min
R.T. 1:50 pm $\rightarrow$ ~2 pm (turning brown)
Cent! R.T. & + 50% acetone at -15° to -20° | Sevofuge
Cent! R.T. & added 100% - - - - -
Flicked & pipetted into suspⁿ. Negligible hemolysis
Back up into 50% for 2 min
- then to PBS 0 $^{\circ}$ C fully denatured by now.
Test - monospacer - look fine.
Cent! & resuspended at 10-30% hematocrit

Anti β^A titrated 1/10, 1/30, 1/90, 1/270 (Blue)
Anti β^S (Black)

5 μ l of each dilution + 5 μ l of ABC
of ab

Incubated at R.T. from 3:15 pm to ~3:50 pm
Washed 3x with PBS, 1st wash showed hemolysis.

2 Controls - no ab 5 μ l PBS + 5 μ l

On test - c'd see no fluorescence, & only remnants of ghosts.

Increase formaldehyde or go to dimethylsuberimidate.

Sat. Oct. 30th

Hard Balls (again)

Ab staining of Hb has 3 phases: —

① Crosslinking	② Permeabilization	③ Ab staining
Hb @ α membrane proteins	of RBC	Find conditions for ab
HCHO	e.g. 50/50 $\rightarrow$ 100% acetone (ice cold)	e.g. de-nature
or DMS $\leftarrow$ O.S. old	Triton etc.	or not at 80°C?

hooking back: —

Ron Jensen	<u>v21 buffer</u>	0.15M NaCl	
	2x osmolar	0.1M Na ₂ CO ₃	
		0.1mM EDTA	pH 10.3 (normal)
		0.01% NaN ₃	- actually 0.1% with HCl

$\rightarrow$ then mix with 11mg/ml dimethylsuberimidate in HCl (i.e. 40mM) to give 20mM final MW 273.2

Expt. on v71 tested DMS ~0.2M, ~0.1, ~0.05, ~0.025, ~0.01250

Pptd. $\nearrow$ Not much difference. $\nearrow$
4 min. at 80° $\rightarrow$ Brown Neatly gone

v75

Double ab stain - spectacular
- can safely use 0.025M @ 4 min at 80°

v151
Good procedure { Washed to ice cold v21 (2x osmolar)
+ equal vol. 0.05M DMS in f.w. + NaOH to ~10.3
37° for 20 min.
Wash 3x PBS
80° for 4 min.
Wash PBS & store. Tested OK on later pages.

Functional Characterization of the Human Hypoxanthine Phosphoribosyltransferase Gene Promoter: Evidence for a Negative Regulatory Element

DIEGO E. RINCÓN-LIMAS, DAVID A. KRUEGER, AND PRAGNA I. PATEL*

Institute for Molecular Genetics, Baylor College of Medicine, Houston, Texas 77030

Received 1 February 1991/Accepted 8 May 1991

The enzyme hypoxanthine phosphoribosyltransferase (HPRT) catalyzes the metabolic salvage of the purine bases hypoxanthine and guanine. We previously characterized the genomic structure of the human *HPRT* gene and described its promoter sequence. In this report, we identify *cis*-acting transcriptional control regions of the human *HPRT* gene by linking various 5'-flanking sequences to the bacterial chloramphenicol acetyltransferase gene. The sequence from positions -219 to -122 relative to the translation initiation site is required for maximal expression of this gene, and it functions equally in both normal and reverse orientations. In addition, a *cis*-acting negative element is present in the region spanning from positions -570 to -388. This negative element can also repress promoters of heterologous genes, such as those of adenosine deaminase and dihydrofolate reductase, which are structurally and functionally similar to the human HPRT promoter. Furthermore, this repressor element functions independently of its orientation but appears to be distance dependent. In vivo competition assays demonstrated that the *trans*-acting factor(s) that binds to this negative element specifically inhibits human HPRT promoter activity. Taken together, these data localize *cis*-acting sequences important in the regulation of human *HPRT* gene expression and should allow the study of protein-DNA interactions which modulate the transcription of this gene.

Hypoxanthine phosphoribosyltransferase (HPRT; IMP: phosphate phosphoribosyltransferase; EC 2.4.2.8) catalyzes the conversion of hypoxanthine and guanine to their respective 5'-mononucleotides and plays a crucial role in the metabolic salvage of purines in mammalian cells. A partial deficiency of the enzyme in humans causes gouty arthritis, as a virtually complete deficiency leads to a devastating neurological disorder, Lesch-Nyhan syndrome (31). The HPRT enzyme is approximately 24.5 kDa and is conserved among hamsters, mice, and humans (4). The functional gene is constitutively expressed in a wide variety of tissues, including cultured cells. Studies of tissue distribution of the enzyme in mammals have indicated that enzyme activity is approximately sevenfold higher in the brain than in other tissues (4, 22, 31). The mechanism for regulated HPRT expression in the brain with the maintenance of a constitutive low level of HPRT expression in other tissues is largely unknown.

The human HPRT (*hHPRT*) promoter is extremely GC rich and lacks a consensus TATA or CAAT sequence, and contains several copies of the sequence 5'-CCGCCCC-3' or its complement (17, 27). Previously, we demonstrated that sequences within 245 bp of the initiation codon were necessary for the transcription of a human *HPRT* minigene. We tested the presence of a negative element upstream of the promoter (28). As a first step toward understanding the regulation of the human *HPRT* gene, we constructed and analyzed a number of deletion mutations within the 5'-flanking region of the gene to define the important *cis*-acting regulatory elements. Our analyses revealed that the sequence from -219 to -122 directs a relatively high level of transcription and also exhibits bidirectional activity. In addition, a downstream regulatory element negatively modulates

this expression. This negative element is able to function in a position-dependent and orientation-independent fashion and is capable of repressing heterologous promoters. The tandem organization of the negative and positive elements suggests that the interplay of these regulatory domains may regulate the tissue-differential and/or constitutive expression of the human *HPRT* gene. Further characterization of the factor(s) that interacts with the negative element should lead to the identification of transcriptional regulators important in the expression of the human *HPRT* gene.

MATERIALS AND METHODS

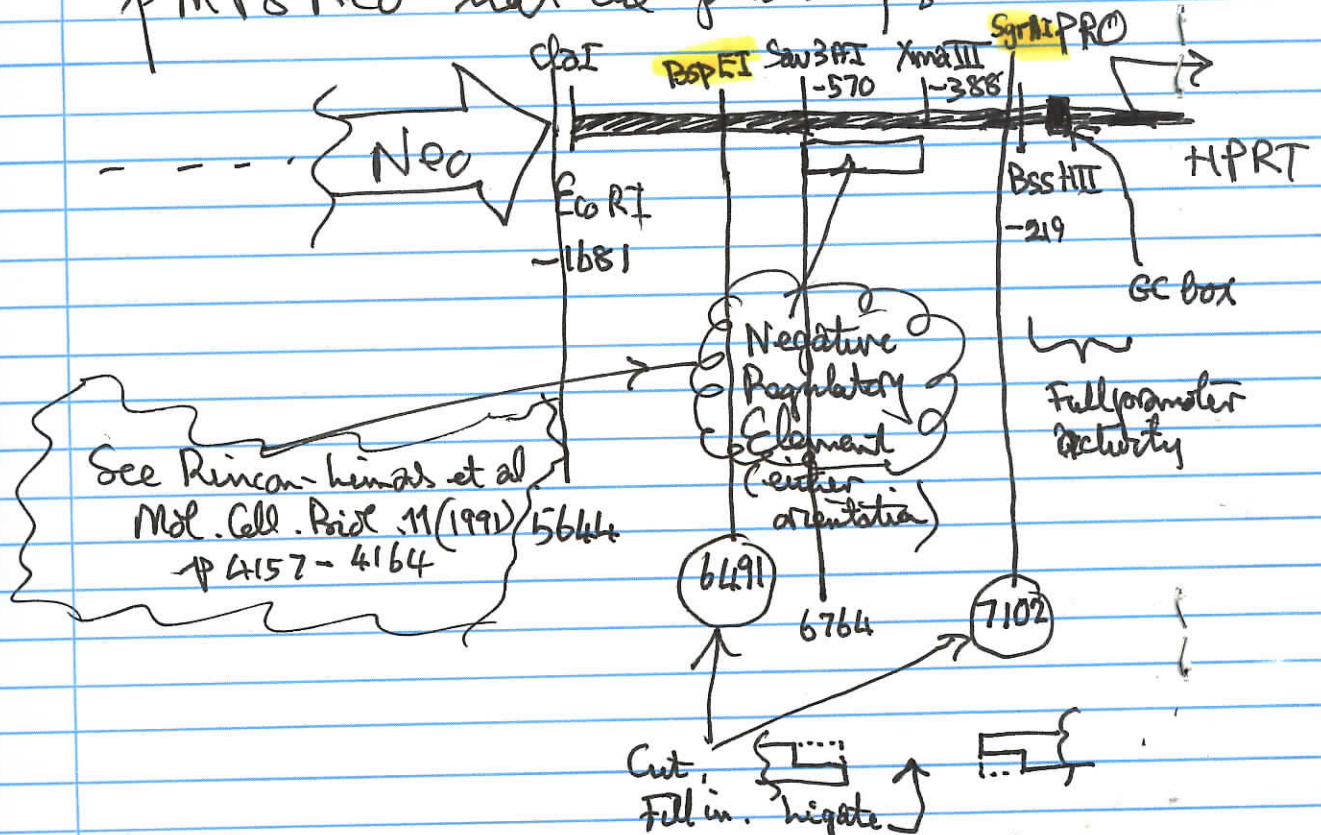
Enzymes and radioisotopes. The Klenow fragment, calf intestinal alkaline phosphatase, and T4 DNA ligase were obtained from Boehringer Mannheim. Restriction endonucleases were obtained from New England BioLabs or Bethesda Research Laboratories and used under the conditions specified by the supplier. Radioisotopes, including [α - 32 P] dCTP, [α - 35 S]dATP, and [14 C]acetylchloramphenicol, were purchased from New England Nuclear.

Miscellaneous expression vectors. pSV0CAT is a promoterless expression vector containing the gene encoding bacterial chloramphenicol acetyltransferase (CAT) (12). All CAT expression plasmids were derived from pSV0CAT. pSV2CAT expresses the CAT gene under the control of the enhancer and promoter of simian virus 40 (12). pXN.8CAT and pEN.24CAT (14) contain 800-bp *Xba*I-*Nco*I and 240-bp *Eco*RI-*Nco*I fragments, respectively, from the murine adenosine deaminase (ADA) promoter driving CAT gene expression in pSV0CAT. pD35CAT (20) has a 297-bp *Nae*I-*Taq*I (-291 to +6) fragment containing the mouse dihydrofolate reductase (DHFR) major promoter inserted into the *Hind*III site of pSV0CAT. pAVE1hGH (29) contains the

Wed. Dec. 15th
pMP8 Neo modification

79
 Had to quit grants etc.
 for experimental
 therapy!

pMP8 Neo has the following structure :-



Modify to remove NRE in view of Seiger's
 small sized HPRT⁺ colonies after targeting
 HPCs & (possibly) HSCs.

pMP8 Neo has been renamed pMP8 NEO
~~pSKB1 following change of function~~
~~side to~~

pSKB1 has a multicloning site
 where Neo is
 EcoRV, R1

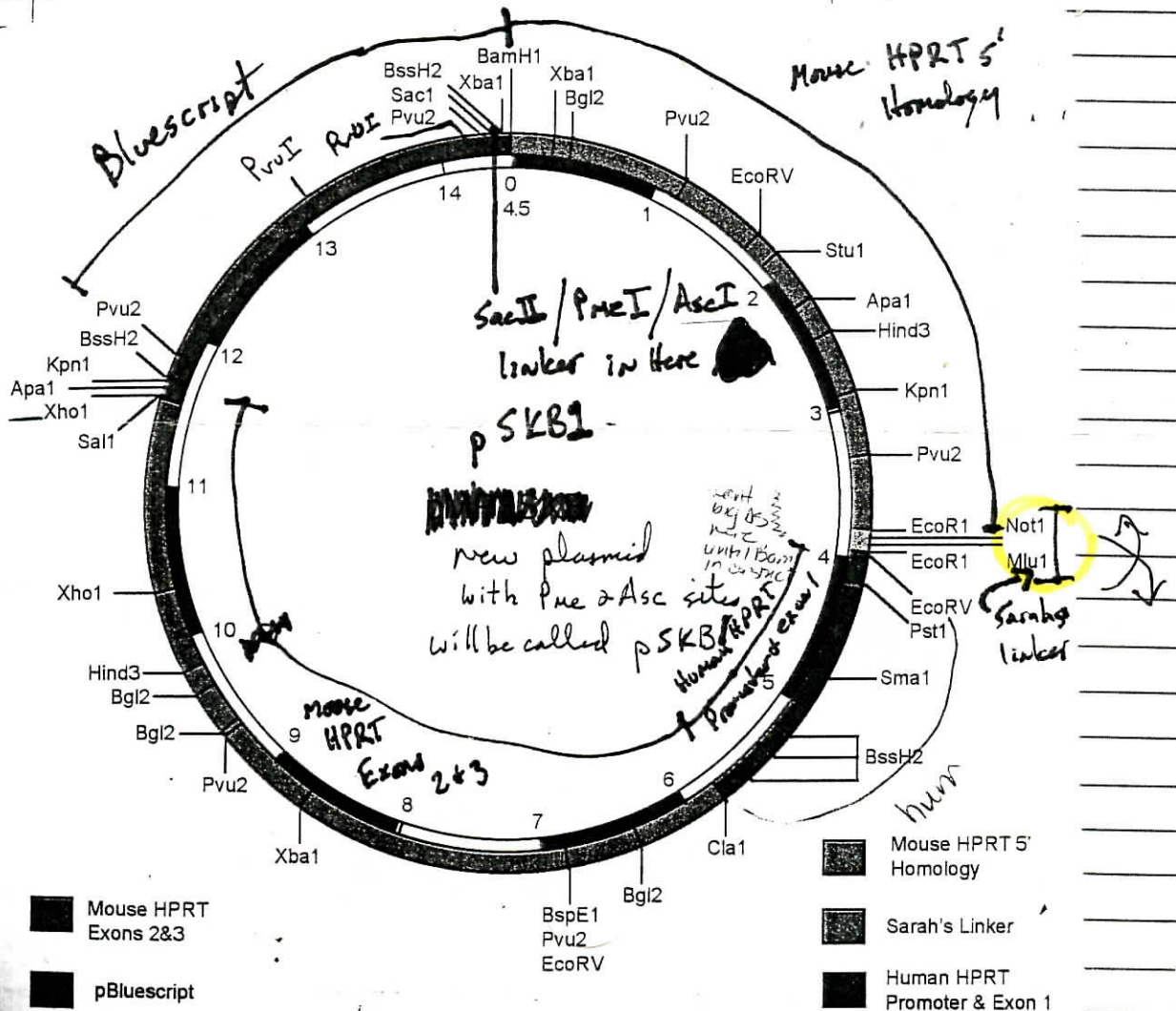
need linker that has different sticky ends
 Blow off XMN linker 35

NMXXN linker

XhoI	NotI		MluI	XhoI	Nde
CTCGAC	GCGGCCGC	ACTTGACATTGCG	ACGCGT	CTCGAGCA	TATG
GAGCT	GCGGCCGC	TGAACGTAAACGC	TGCGCA	GAGCTC	GTATAC

SPAB vector for putting in additional 8 base pair cutters

SacII	PmeI		AscI		BamHI
CTCCACCGC	GGA	GTTTAAAC	CGT	GGCGCGCCG	GATCCCCCG
GAGGTGG	CGCCT	CAAA TTG	GCA	CCGCGCGCC	CTAGGGCC



N.B.

SPAB linker was put in the SacII-BamHI site of pSKB1 to yield pSKB1.

PmeI & AscI can be used to linearize this plasmid for getting homologous recombination HPRT

Thurs Dec. 16th

PSKB1 modification

J'81 pSKB1 from K.C. 1.7 ng/ μ l
SGM / KC
Remin book
pg 35

Take $\left\{ \begin{array}{l} 5 \mu\text{l NE buffer} \geq 10\times \\ 42 \mu\text{l dH}_2\text{O} \end{array} \right\}$

$\checkmark 3 \mu\text{l (5 } \mu\text{g)} \text{ J'81 pSKB1}$

$\checkmark 2 \mu\text{l (20 } \mu\text{l)} \text{ BspEI hot 2 (1992)}$

J'81
SKB
Bsp ~0.1 $\mu\text{g}/\mu\text{l}$

(10 $\mu\text{l}/\mu\text{l}$)

37° 4:37pm to 5:30pm

to 6:45pm

2+15
Post
J'81

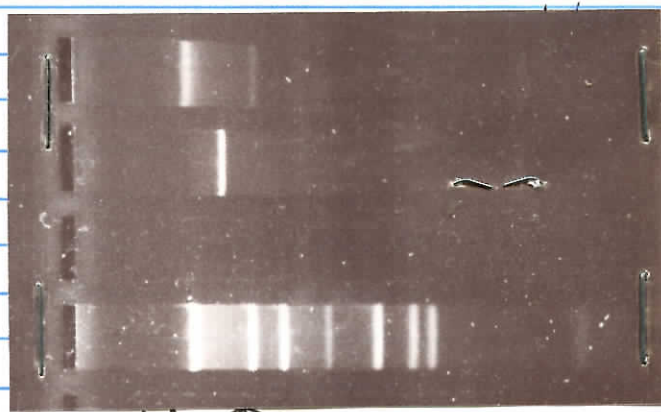
①

ON 5:40pm
R 6:25pm

0.86 2+15
Hx4 2+15
max.

2+15
Pre
J'81

Dye
W
Pre BspEI
Post
W
AgMM
W
Dye



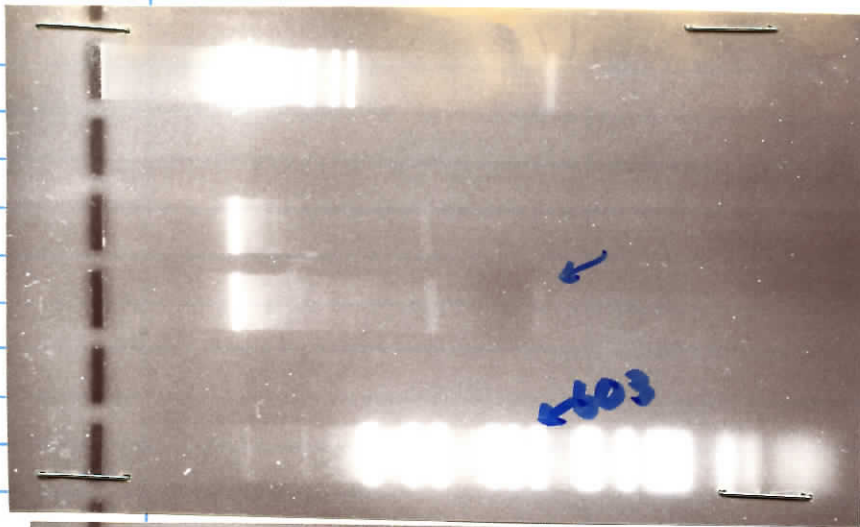
Conclusion . Good cut . Proceed .

$\therefore + 5 \mu\text{l SGM} + 133 \mu\text{l EtOH}$

Freeze . Cent . Dry . Back into 40 μl ~~ESTABLISHED~~

J'81
SKB Bsp

①

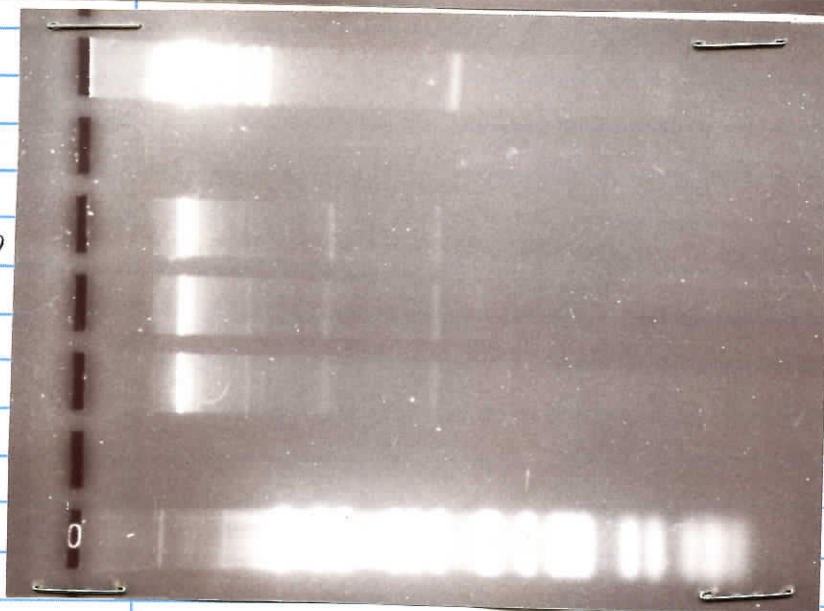
① Conclusion

Just detecting the
606 bp fragment.
Either env. poor or
conc. too low.
Repeat & retest.

①

0/

②

② Conclusion:

Same increase in layer
time &/or with new
buffer. Aat only
just started. Freeze
all & proceed
with I'83 SKB. Sqr²

g+10

②

Rel. h₂o
Esp. &
Sqr

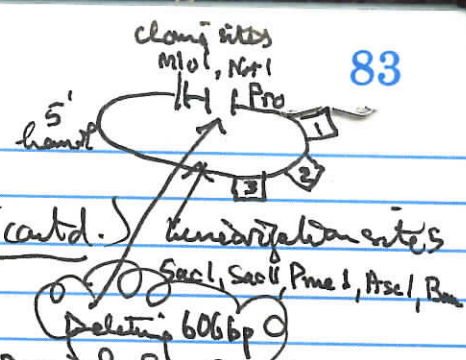
g+10

11

11

Fri. Dec. 17

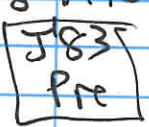
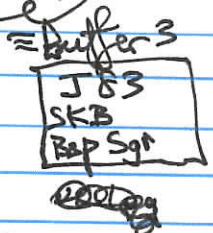
SKB modification (contd.)



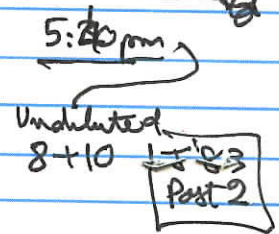
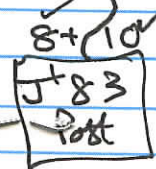
1:20pm
1:50pm
M.570 HX
10v/cm

Dye
W
Pre
Post
W
AclMM
W
8

Take 20 μ l ($\approx 2 \mu$ g) J'81 SKB (post Bsp)
+ 6 μ l 10x RS+1M NaCl
+ 34 μ l ddw
+ 1 μ l Sqr AI (10v) Lot #2 (10v/ μ l)

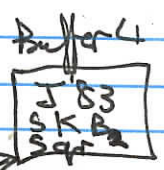


37° 11:56 to 1:05pm



New England have changed their buffer preference!!

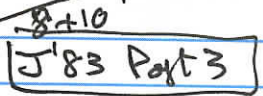
So take 10 μ l ($\approx 1 \mu$ g) J'81 SKB (post Bsp)
+ 3 μ l 10x Buffer 4
+ 17 μ l ddw
+ 1 μ l Sqr AI (10v) Lot 2 (10v/ μ l)



1:55pm
2:25pm
M.570 HX
10v/cm

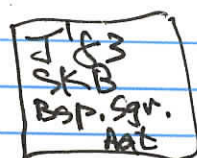
Pre
W
Agmm
J'83 SKB Bsp Sqr Post 2
J'83 SKB Sqr 2 Post 3
J'83 SKB Bsp. Sqr. Acl Post 4
W
Acl. MW
W
Dye

37° 4:05 to 5:40pm



Can add Acl II as a safeguard for next step - it is a single site cutter within the deleted region.

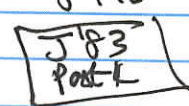
took 15 μ l of ~~SKB~~ and added 1 μ l (4v) of Acl II Lot 3 (4v/ μ l) also buffer 4

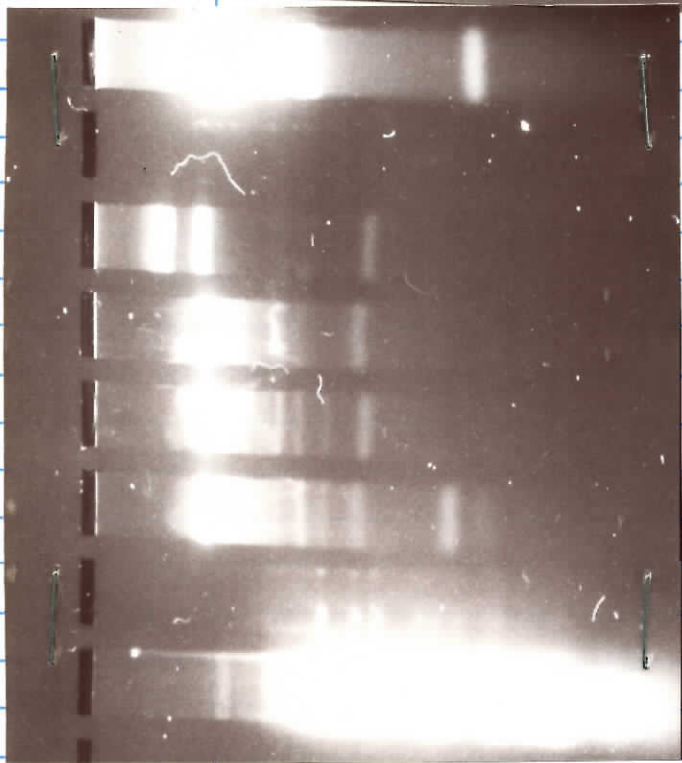
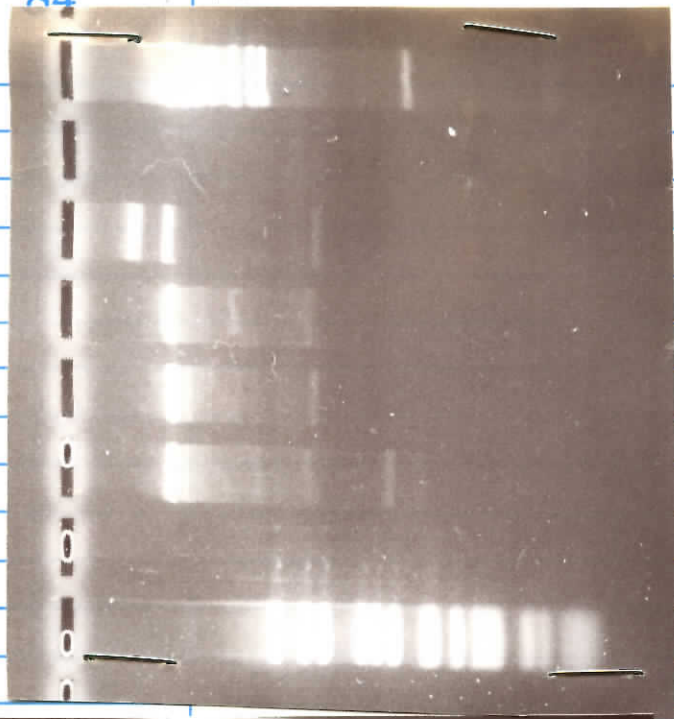


4:45pm

to 5:40pm

8+10





Sat Dec. 18th

Deletion in SKB1 (fresh start)

A better scheme for two enzymes that probably can work in the same buffer (test R1 salts typically is:-

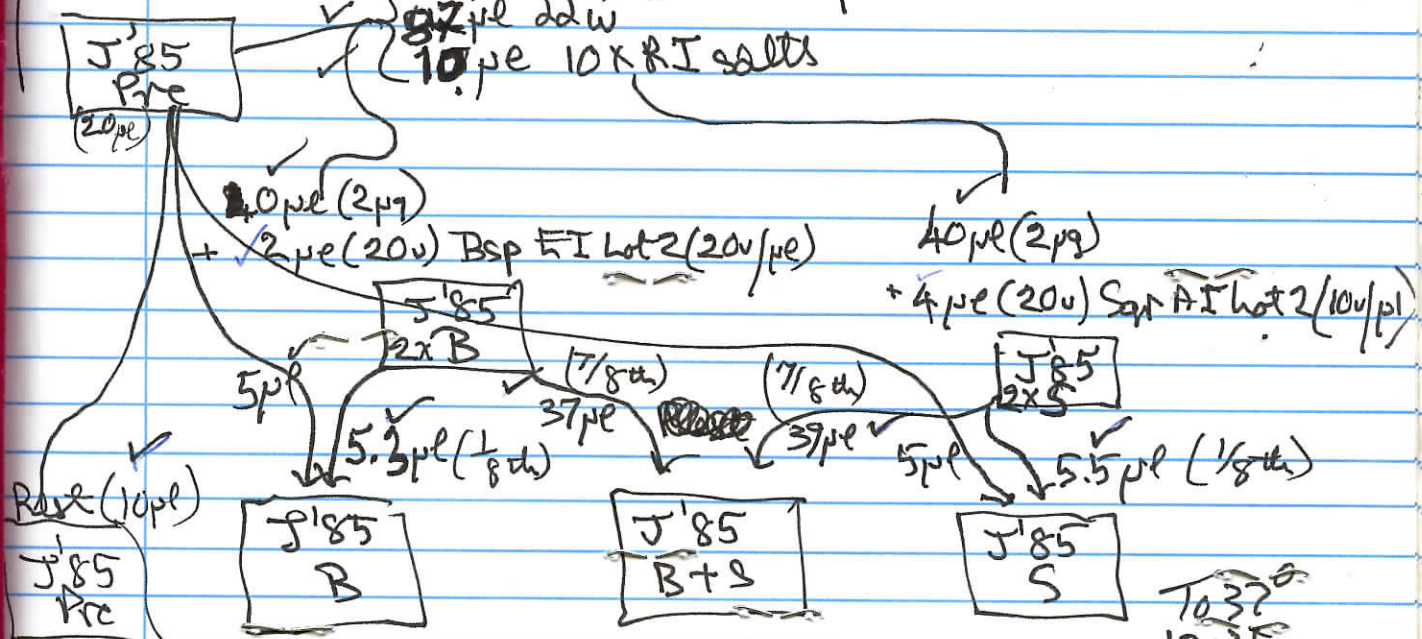
DNA in buffer 0 100%. Add enzyme A to ^{at 2X} 40%
Add enzyme B to ^{at 2X} 40%
5% + 5% 5% 5% 35% 35% 10%

Mix A+0, B+0, A+B & use 0 as pre.

Bye All enzyme to substrate ratios & DNA conc. are same.

∴ take 3 μ l (5 μ g) of 81 pSKB1

87 μ l ddw
10 μ l 10X RI salts



Test all at 8+8 in 1.5% Hx1

To 37°
12:35 pm

3:24 pm

OB 102 $\equiv$ 5.1 pg / pl

Orig Name:

← J'87 Blue hunker

G	C	C	G
G	A	A	A
A	G	A	A
C	G	T	T
C	A	A	A
C	C	T	T
A	G	G	A
A	A	C	T
T	C	G	T
T	G	A	A
G	G	C	C
C	G	G	G

3

C	C	A
C	G	
-	-	-

24663

IFT Radl. L.

2. Original Name: G. T. Peanurka
 C C G G C C G T G G C C C G G C C C
 T T C A C G T G T T C A C G A
 G T T C C T A G G T T G A C G
 T C T T

Can also
go into
AVRDM. seg
(see p. 12)

New downing sites
for PSKB1.del

Old cloning sites
for pSKB 1. del

linearization sites
for pSKB1.del

Enzymes that do cut and were not excluded:

AotII	ApolI	AscI	AvrII	BamHI	BspEI	BsrDI	BssII
BstEII	ClaI	FseI	FspI	HgiEII	MluI	MunI	NarI
NorI	NsiI	NspV	PacI	PmaI	PmeI	PmlI	PpilI
PshAI	PvuI	SacI	SacII	SgrAI	SmaI	SpeI	StuI

Enzymes that do not cut:

improved SKB1 [or AVR DIM (see J'12)]

Put in a linker with max^{im} clamping rates:-

Sma I ~ can ~~accept~~^{accept} blunt ends

Bsp CI - Age I, Bsa WI, Bsa FI, Sgr AI, AVal I, $\frac{1}{\text{Small I}}$

$$A_{at} II - T_{aa} I$$

Aut II - Nhe I, Spe I, Xba I, Sty I

BS, SI - No other!

Prob I - blunt ends

Fse I (8 base) blocked by CpG meⁿ. - No interest

Sart AI Agt I, BsaWI, BspFI, BsfI, NgoMI, AarI
XmaI

For pointing in two insulators from 5'64,
best bet may be to single cut pIC13-1 with
Xba I & ~~fragments~~ blunt ligate, then find clone -
that gives 2-4 kb insulator, or use single
insulator Xba I. Meanwhile a simpler
oligo will be 5'87 Xba linker for use with pSKB1

Oligo # 5 Oligo Name: J84 Xba linker 3'
 Date made: CICIG GAAA CICIT AGIG TTT
 LENGTH: 14
 % G+C: 57%
XbaI ~~_____~~ → ApaI ~~_____~~

With 8 enzymes: SMAI BSPEI AATII AVRII BSSSI PMLI FSEI SGRAI

December 19, 1999 16:37

BspEI AvrII

GATTCTCCTGCCTCAGCTccggaacctagggttCCGGCGTAGGCG

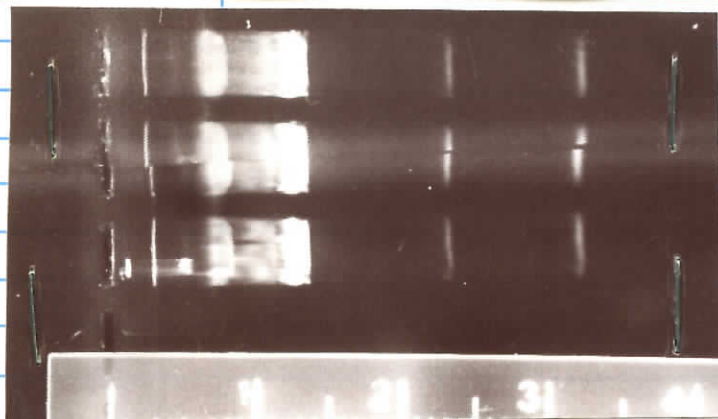
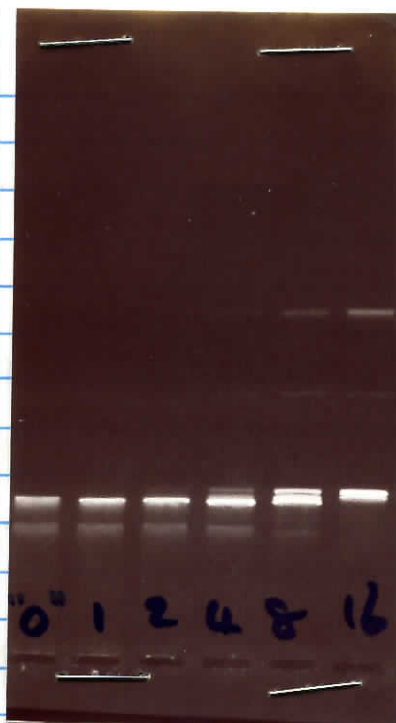
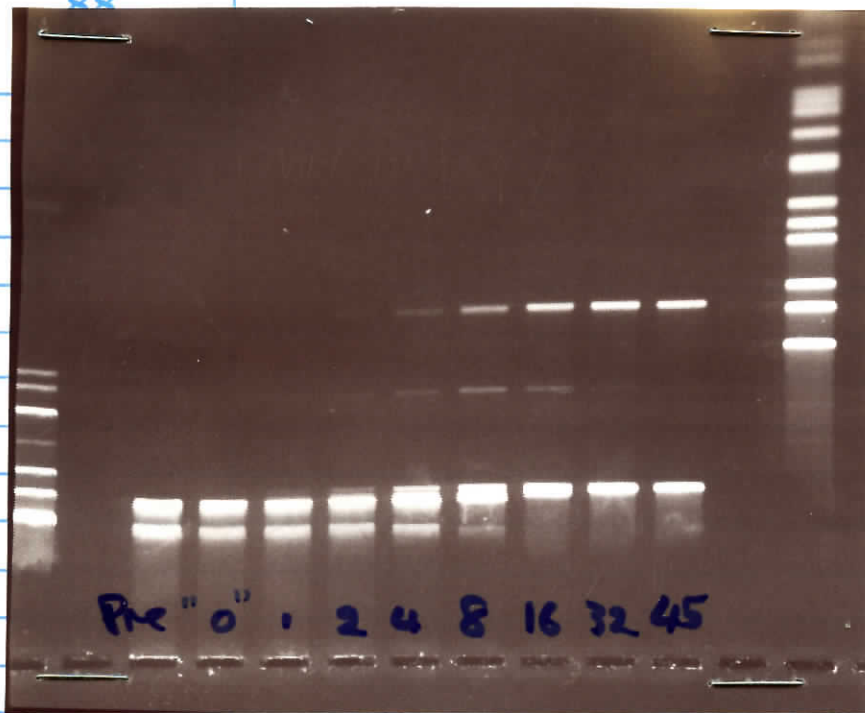
CTAAGAGGACGGAGTCGAagccttgatccaaGGCCGCATCCGC 44

999 16:37 ..

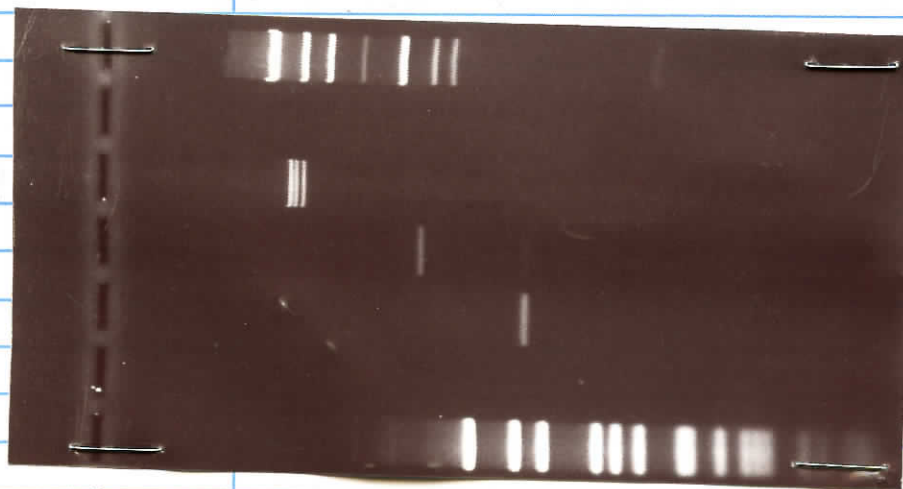
Accepts ~~XAI~~, leaves ~~AspEI~~,
removes ~~SgrAI~~

GGCGTAGGCG

(1)



(2)



(3)

(3) 1.27 Hx1
 1.500 max
 2.226

Dye

W

ACM

W

S

M

F'

W

ACR

W

Dye

10+6

10+6

10+6

(3)

Enrichment: nice products! Proceed!

5 is single cut, minus 1 silencer & minus 2 silencer
 M mainly 2.4 kb

on 1.2 kb max ~ 30 min

Dye
W
Pre
"0"

Sun. Dec. 19th

89
Xba Xba Xba
insulator insulator...

Single cut Xba JC13-1

↑
Priming
stud,

Take 7 µl (10µg) J'65 JC13-1

445 µl ddw

50 µl 10x RI salts

J'89
Xba

I 153
protocol

Prewarm to 37°

+1 µl (20u) XbaI lot 30 (20u u/µl)

10 µl + 10 µl PBS

"0"

1

2

4

8

16

32

45

130 µl + 130 µl

37-38.5°

incubation

The PBS may have
been contaminated
- but 380°
anyway.

50 µl
to

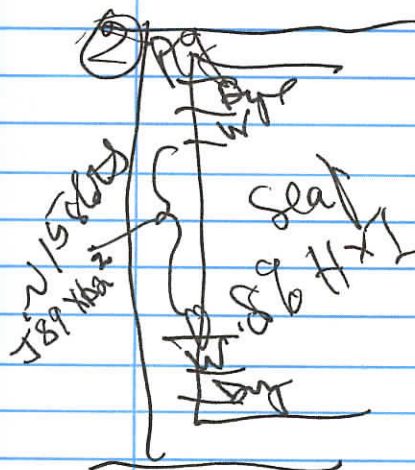
50 µl PBS and 80°C for ≥ 10 min.

incubation

All frozen.

Fill in recipe on J'7

- (1) Conclusion Nice series. Tempting to go for 8 min. → isolate the 2.4 kb timer. The 4 min. single cut might be reachable. The 1.2 kb monomer is likely. Could take a chance & set up repeat of J'89 Xba² for 6 min. → try to isolate as many as poss. in 8% HX1. Inactivate at 80° without PBS. Temp was ~36.5°



on 6:27 pm → to 2V/cm at 6:50 pm

off 6:33 pm

6V/cm (rough)

Back to 6V/cm at 8:15

Back into 0.001M EDTA

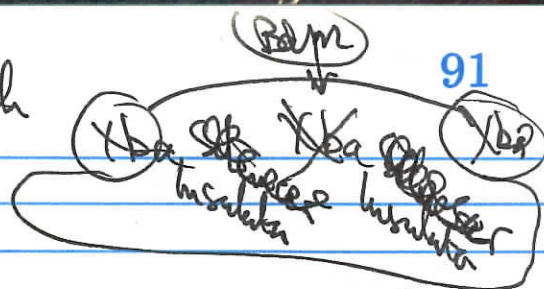
J'89 F	30 µl	(1.2 kb)
J'89 M	30 µl	(2.4 kb)
J'89 S	120 µl	Vector

J' 91² picked 24 to 32° with shaking at 5:20pm Dec 23rd

2374

Mon Dec. 20th

Silencers



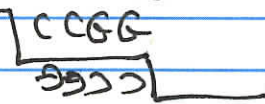
91

Nice way to put two silencers into pSKB1
 is to use Xba → Bam linker. half
 of the Bam linearization site is only minor
 problem (non-existent) & detection can be
 by Bam or Xba (many pieces in pSKB)

Tues. Dec. 21st

pSKB1 deleting silencers (contd.)

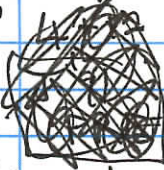
Simplest way is to take the BspFI / SgrAI
 digest from 5'83 → 5'85, dilute, ligate
 & plate. The two sites have
 overlap & don't give back
 either so can diagnose
 with mixture & R1220.



Take 100 μl pL ddw ✓
 2 μl 15'85 B+S ✓
 2 μl 10X p95 buffer
 + 2 μl Taq polymerase
 dilute hot 970 10 μl

91
 LIGN

O.K.!



Repeated.

12/22 work 12/3 cells J91

J91 Red 12/19

Suspicious date
 Discarded 12/22

15' 9:56 am to 1:10^{pm} R.T. 1:10^{pm} to 2:15^{pm} 37° 2:15^{pm} to 5:30^{pm}

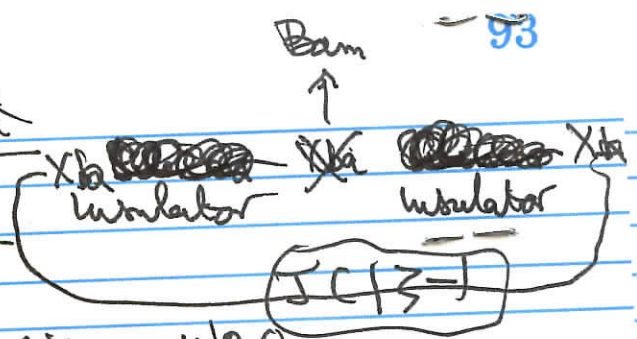
Transformation

80 μl x NZY Amp Rxt 20, 5, 20, 5
 8 μl x 37° 6:30^{pm}
 40.5 J91² 11, 0 am -

No colonies

Tues. Dec. 21st

Single cut Xba → Bam
on JC 13-1



Use J'89 Sphus Xba → Bam linker H'20

- Take 10 µl J'89S
- ✓ + 2 µl p95 10x ligation buffer
 - ✓ + 1 µl H'20 Xba → Bam linker
 - ✓ + 4 µl ddw
 - ✓ + 2 µl T4 ligase to cold 5:52 pm

Transformations

3 DH5α J'89 50 µl
J'93 ligⁿ Blue 8 µl
J'93 ligⁿ Red 8 µl
0° 9-50 min - 10-40 min
42° 90 sec

Plate R, 20, 5, 20 / 16, 5 / 16

37°

(15) Blue

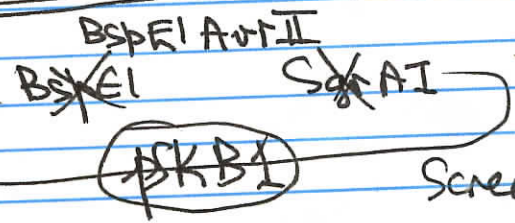
Hold pending
J'93 Red & use
of J'89 N

Screen with Xba

J'93 Blue
LIGⁿ

(6) Red

PKSB1



Deletion of Avr II site

Screen with
Avr II

Use J'85 B+S plus "Xba linker" J'87

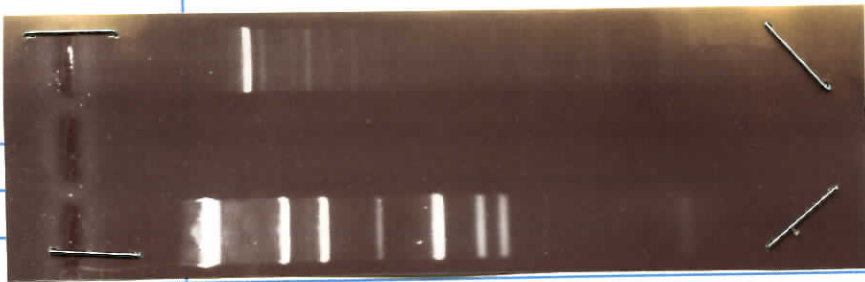
- Take 2 µl J'85 B+S
- ✓ 2 µl p95 10x ligation buffer
 - ✓ 1 µl J'87 "Xba linker"
 - ✓ 15 µl ddw
 - ✓ 2 µl T4 ligase Lot 970 to cold 5:52 pm

Picked 24 & 6

37° at 5:20 pm Dec. 23rd

J'93 R Red
(1) - (24)

J'93 Red
LIGⁿ



(1) Conclusion:
Kinn was correct!
- heat did not hurt

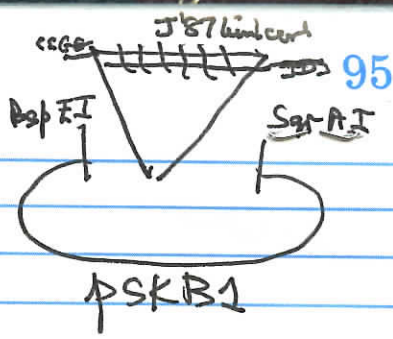
① 2
02

6+12

3:26pm
4:26pm
0.86 HX4
NAX

Wed. Dec. 22nd

linker insertion



Dye
W
H2 J'85 B+S post-heating*
W
HGMN
W
Dye

The protocol on D'39 was very successful (~20%)

I took ~1 to 2 µg of cut plasmid (not CIAPed) + 4 µg of each linker in 20 µl p95 1x + 4 µl conc ligase TK. 15° 2 hr, RT 1 hr, 37° 1 hr. Then to 8 µl + 0.9 µl 10x PBS under oil at 85° in PCR machine & slow cool (pump off) from 85 - ~50° 80 min. & used whole with 80 µl DH5d for transformation. D'47 second linker insertion was less good (~4%). Pre & post samples were 1 µl pre & 2 µl post. Note I did not dilute before the annealing much.

J'95
LIGⁿ

take 30 µl (~1.5 µg) J'85 B+S heated*
+ 3.3 µl (~4 µg) J'87 Blue linker
+ 0.8 µl (~4 µg) J'87 Red linker
+ 4 µl 10x p95 buffer
+ 4 µl T4 ligase (white) lot 970 (1u/µl)
heat inact. 80° for 20 min
Cpt up to 89°!
bitter check - Kim says O.K.

15° 2:37 pm to 4:25 pm RT 4:25 to 5:25 37° 5:25 to 6:05 pm

10 µl + 0.1 µl 10x PBS under oil 85° 6:10 to 5:53 at
6:30 pm < 75° 6:10 pm 85°

8 pm 41° Transformation

J'91	DH5d	80 µl	Plated	2x Amp NZY
J'95 lig ⁿ		8 µl	R	20, 5, 20, 5
0° 8:12 to 8:47 pm				16, 16
90 sec 42°				

37° 9 pm
- by noon - no colonies.
Repeat without the slow cool & also with less DNA



①

Conclusion:

No candy bites.
Hold until later.

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Thurs. Dec. 23rd

Repeat of 8'95 linker ligation

Christmas Eve Transformation

J'97 Blue 37° 2:10pm → 11am
 J'91 DH5d 80µl ✓ R 20, 5, 20, 5
 J'95 (cold lig) 8µl ✓ (33) (7) (3) 16, 16
 0° 12:30pm to 1:27pm (Amp² XN2Y) 57, 16
 42° 90sec. 1:40pm
 11am

~~Negative~~ Try less DNA; there's a lot of oligo.

12:52pm to 1:27pm
 J'93 J'91 DH5d 80µl ✓
 J'95 lig + PBS 1µl ✓
 J'95 beat/slo cool
 but less
 Plated R 20, 5, 20, 5
 16, 16
 Still no colonies.
 11am

Sat. Dec. 25th

Take 90µl 10X RI salts

✓ 765µl ddw

✓ 45µl (180v) Aot II Lot 9 (4v/µl)

38µl plus excision J'99 to each 2µl minus

On to 37° 2:30pm to 6:30pm Fryer

J'97 (1) - (24)
 Blue

(23)

Also insulator dimer insert 99

Sat. Dec. 25th (Christmas Day)

12:15 pm
1:25 pm
0:50 Hx4 Max

Bye
W
AGMM
W

hinker

pBSK I. del
+ "Xba linker"

Espeii
pSKB1
AvrII

Take 100 μ l DRI salt
850 μ l ddw
50 μ l AvrII (4v/sample) lot 29 (4v/ μ l)

J93R
mini

38 μ l $\times$ 24 + 2 μ l each J93R(ed) mini
37⁰ 11:50 am to 11:50 — 2 pm
But this was not correct!!

24
MM
MM
Dye

Recovered 98 μ l from (1) (7) (19) & (23)

+ 25 μ l 5X CIAP buffer
+ 2 μ l lgb CIAP

Red
J99
AVR CIAP

55⁰ 2:43 pm to 3:13 pm

6:30 pm 1X DPH/400
2X CAA

insulator

Take 3 μ l J99 AVR CIAP
+ 15 μ l J89M (6 μ l left)
+ 2 μ l 10X p95
+ 2 μ l T4 ligase lot 970 (1v/ μ l)

J99
LIG^N

Avr Kba Kba
insulator AvrII
pSKB del

6+12
J99 PRE

15⁰ 6:55 pm to 8:15 pm Cold 8:15 pm to next day

6+12
J99
Post

#1 (lpe) Safety
- Antibiotic
- abt yet

Transformation J99 Red

J91 DH5 α
J99 LIG^N

80 μ l
8 μ l rest
27 μ l

Plated NZY 2X Amp

0⁰ 11:46 am to 12:22 pm

5:30 pm

13 (3) 16, 16 (2)
3209 pm to 10 am

420 90 sec

2 rows
for 1/2 hr
6 am!!

J99 Red minis (1) (24)
37⁰ 4:30 pm Mon. Dec. 27th to

MluI

NotI

4501

4560

4561

4620

4621

4680

4681

1740

891 bp

5221

5280

SmaI

5281

340

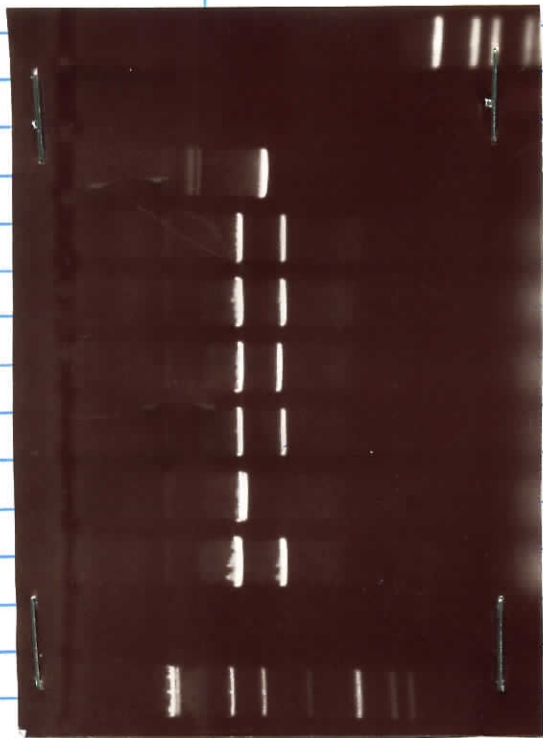
BspE

AvrI

5'101 AVR Red

5341

00



① Conclusions: Didn't pick them correctly - all are too big. ~~Say~~ rest & try again next millennium!

Wrong interpretation
see J107

J'99 #11 proved correct



J'103 Blue 2

Oligo Name: J'103 Blue 2		3'			
24875	CITG	AAG	TGIC	AGIT	GGIT
ndc:	GCG	ATI			
File: 20					
D: 55%					

103

Ant-II

Xba

insulator

Xba

Ant-II

Xba

insulator

Xba

Ant-II

103

840 W (6) (8) (9) (11) (12) (21) (23) J 99 min

ACR $\overline{mm}$

W



12

⑤

2

2

(12)

(21)

(2)

W

Ag-M-M

W

Ans

✓ 136 pl Ldw

16 pt 10x RISalt

8_{pe}(40u) NheI Lot 23 (5u/pe)

20 pl + 2 pl = 5'99 Red minus

(except $\ln$ for #6)

37 ⁰ 9:15 ^{am} to 11:10 ^{am}

Correct will be ~~7.6~~ 7.9 kb

Starting " " 0.0 & 5.5 kb

Monomer " " 0.046.7 kb

? are these correct?

Booth's check
599 (19) by
sequence →

(hunger is there, but beyond is?)

Sequence checks at only fair level with J103 Blue. Try again with J103 Blue 2. Seeds checked length against statting planned J81 pSKB1 - it is okay. Proceed anyway.

J'103 Blue
120µm J-60

Sengo, was into 300 pl

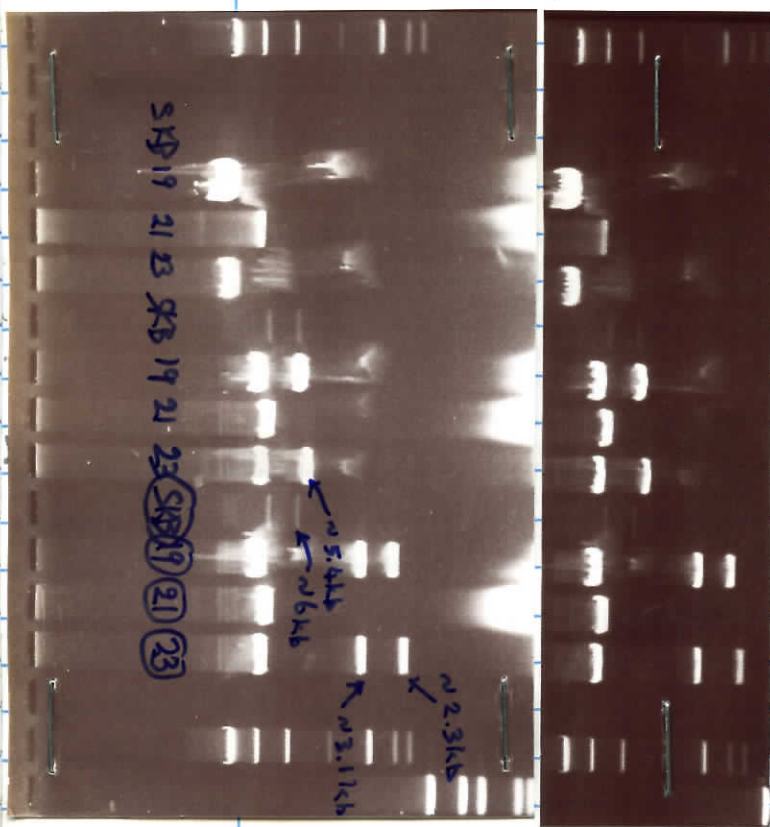
J'103 Blue / 100

56 μ e J1038 blue 120 μ m J-60
+ 994 μ e 0.001 M EDTA

5' Oligo Name: J 103 Blue

Code: <u>1</u>	<u>T G T T</u>	<u>C G C C</u>	<u>G G A A</u>	<u>G G C C</u>	<u>T G T A</u>
Tit: <u>18</u>	<u>A G T T</u>	<u> </u>	<u> </u>	<u> </u>	<u> </u>
<u>61</u>	<u> </u>	<u> </u>	<u> </u>	<u> </u>	<u> </u>

[illegible]



Conclusion:

PSKB1 has no Aot2 site, as expected & two Nhe2 sites!

(21) has no Aot2 site as expected. May have insulator

(19) & (23) have Aot2, so cannot have insulator.

Butter Nhe I digests of J'99 set on page J'102

①	07/12/10	Dye
	98	W
	99	AGM
	0	W
	98	SKB
	93	(19)
	99	(21)
	99	(23)
	81	SKB
	93	(19)
	99	(21)
	99	(23)
	SKB	
	(19)	
	(21)	
	(23)	
	W	
	AGM	
	ACR	
	W	
	Dye	

Recollecting that the insulator insertion was attempted on pooled Aot linker colonies J'99 (1) (7) (19) & (23), it is possible that J'99 (23) is correct Aot linker, and that J'99 (21) contains the insulator. J'93 (19) is too big to be Aot linker. Try sequencing J'99 (21) & (23) - ops (11).

~~Insulator insertion attempt on Aot linker colonies J'99 (1) (7) (19) & (23) failed. PSKB1 has no Aot2 site, as expected & two Nhe2 sites! (21) has no Aot2 site as expected. May have insulator (19) & (23) have Aot2, so cannot have insulator. Butter Nhe I digests of J'99 set on page J'102~~

Millennium 2000 celebrated
in London on Greenwich meridian.

Jan 16th

Recheck on pSKB1. AVR II etc.

AVR II

Nhe I

Nhe I
+
AVR II

Take 14 μ l 10X RI salts
118 μ l ddw
7 μ l (350) Nhe I lot 23 (5 μ l)

14 μ l 10X RI salts
118 μ l ddw
80 μ l (320) Avr II lot 29 (40 μ l)

MIX ANO

20 μ l portions

	MIXA	MIX N	MIX AN
Diluted 0.8 + 4			
J81 1000 1 μ l	81A	81N	81AN
J93 Red min (19) 1 μ l	19A	19N	19AN
J99 min (21) (2 μ l)	21A	21N	21AN
J99 min (23) (2 μ l)	23A	23N	23AN

← sh'd be labeled 81

37°C 5 pm to 8:15 pm
(cold)

pSKB1. AVR II 7700
Nhe I Cut at 2292
Avr at 54081

5365 to cut this

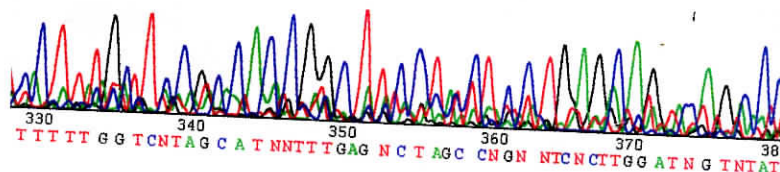
(5.4) ~~into~~ (3.1) + (2.3)

7700
5365
2335

5365
2292
3073

J'81 should be + 606 in place of (5.4)
(6.0 kb)

The "cut out" silencer should
be 608 bp



AVR II

71 m 142

cgggaacatggttccgcctgatagcaccgcccgcccccccaggccttcacccggrrr
5360 +-----+

gcttggatccaaaggcccatcgcgcccgcccccccggcccccgaacacccccc
-----+-----+-----
5419

CCCCCCCCCAGAGGCGGGCTGCTTTTAACTTCAG

5479

CTCAGGCGAMTTTTCGGCTTAAAT-
AAGAAGACGCTGCTCGG

[illegible]

5339

BSHII

[illegible]

GGAGACGAGCGGTGGCCGAGGAGGAGATTTCTATCCCCCCCCC-----5599

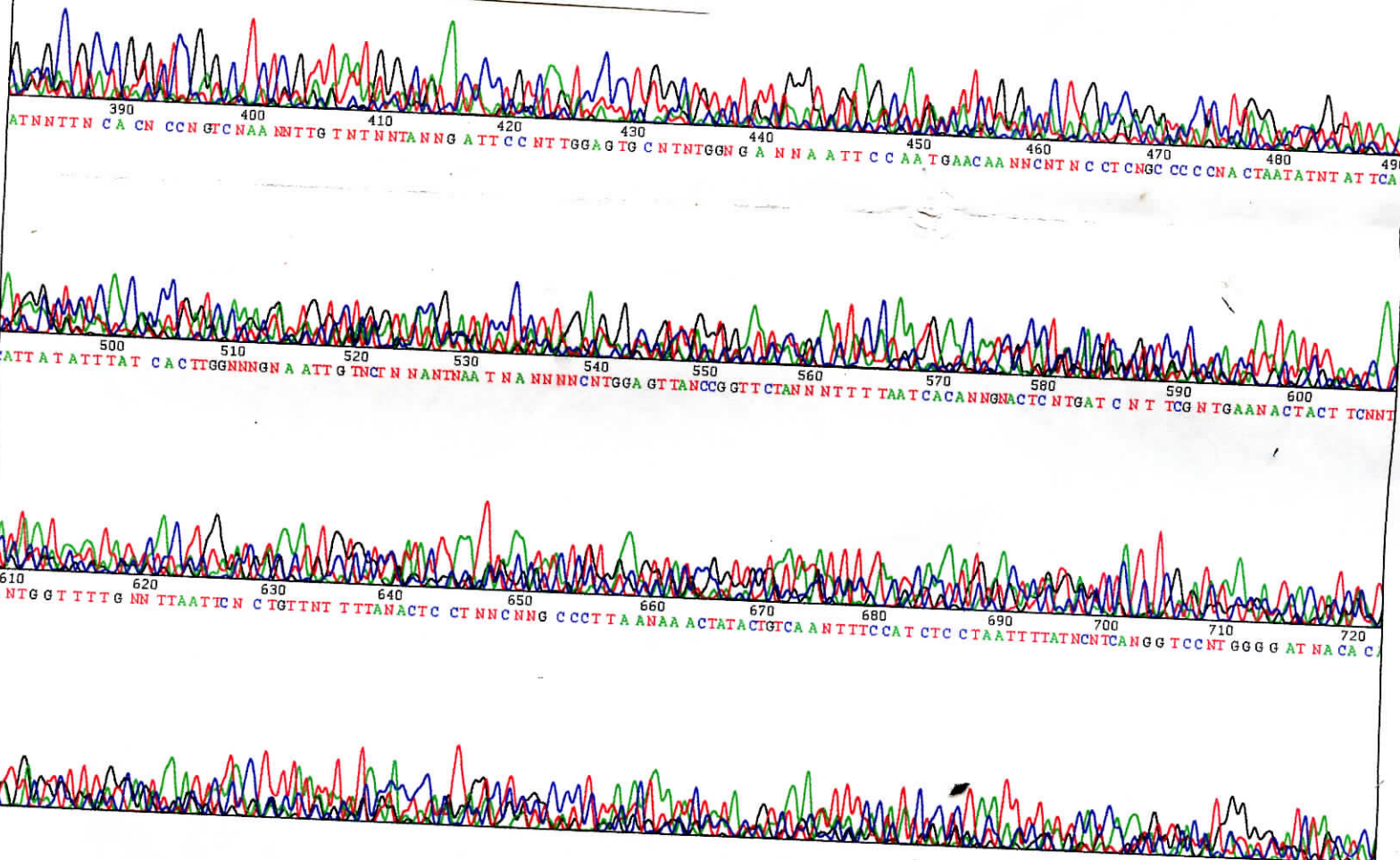
[illegible]

5' - GAGCAGCTCGGCTGCGGCGCTGCGCG - 3'

5659

ind $\frac{\text{spare}}{\text{part}}$ / part

FNRH



Tues. Jan 18th

Sequencing for AVR II etc.

For submitted Jan 18th
Sequences

	How	Template	Primer	
Seq 140	13 pl ✓	2 μ l J'99. min ✓ (11)	J'103 Blue 2 5 μ l ✓ 100	18 pl
Seq 141	13 pl ✓	2 μ l J'99. min ✓ (21)	J'103 Blue 2 5 μ l ✓ 100	
Seq 142	13 pl ✓	2 μ l J'99. min ✓ (23)	J'103 Blue 2 5 μ l ✓ 100	
Seq 143	13 pl ✓	2 μ l J'99. min ✓ (23)	J'103 Blue 2 5 μ l ✓ 100	(1)

52 μ l ddw
20 μ l
J'103 Blue 2
100

Sequence 140 is correct!

Sequence 141 ~~is not correct~~

Sequence 142 & 143 have

has silencer, but could be
wrong deletion so that it
deleted too much *

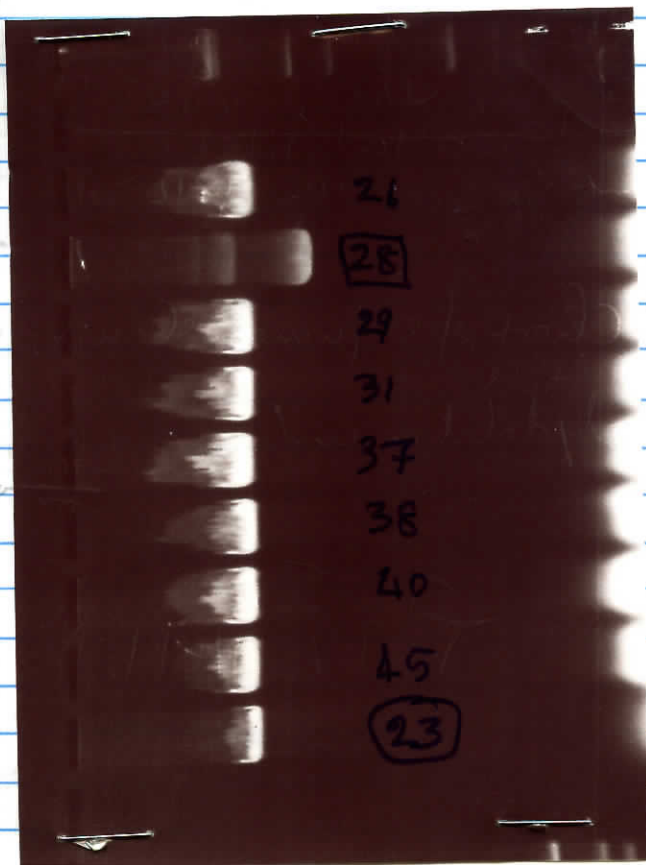
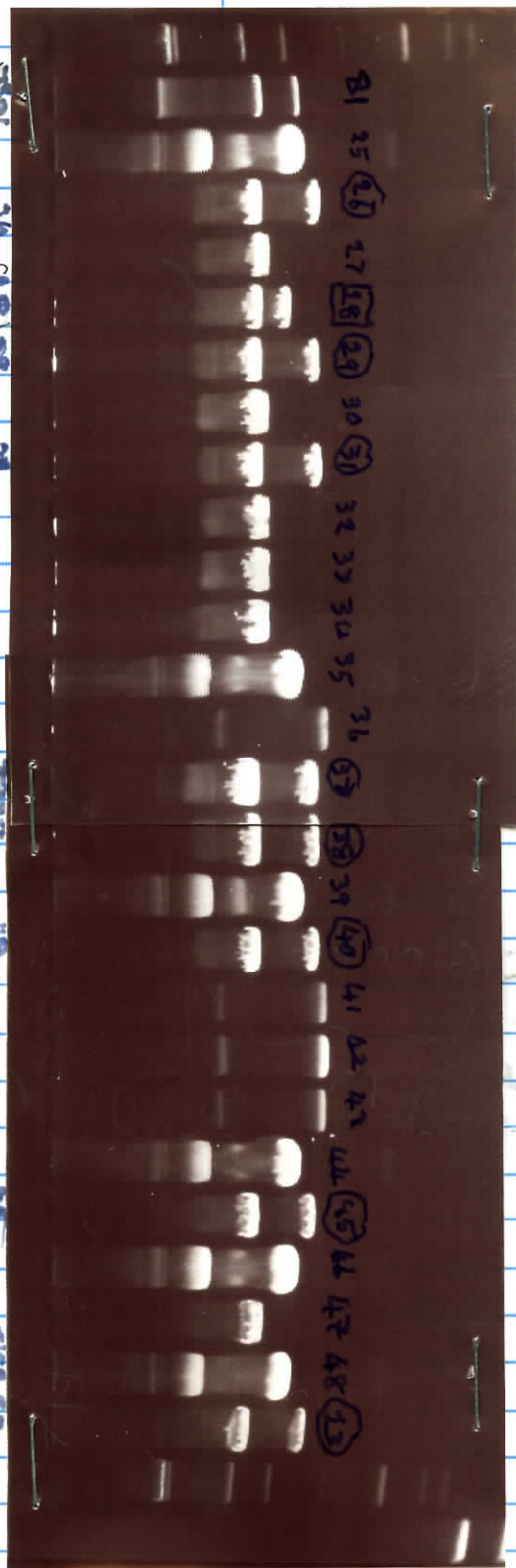
* Check of sequence shows a C C C G G e
at deletion end.

Close to Seq AI but ~~should~~
should be G.

J'99 #11 to Seqgo
Red

for growth - PSKBL AVR II

J'107. PSKBL AVR II



① Conclusion:

7 look equivalent to J'99 (23)

6 look J'99 (21)

#28 ~~7~~ looks v. promising for 2 Lns

However, only safe way is to use a clear AVR II and then add circles. Wait for (23) sequence, except for checking AVR II in the candidates.

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05:55 pm
 0.56 Hx4
 3 pl 10 ml
 RNase
 max

↓ to 1/2 at 6:55 pm. to eat. (<3V/am) off 8:25 pm

Tues. Jan 18th

Reformat J'99 lig.

Dye

AGMM

J'81 pSKB

(25)

(26)

(47)

(48)

J'99 (23)

AGMM

ACRMM

J'99²

J'91 DH5d 80 pl
 J'99 lig. ~7 pl (ret)
 0° 5:30 pm to 5:50 pm

42° 90 sec.

Plated 1xNZY R, 20, 5, 20, 5, 16, 16
 37° 1 pm - 1 pm

J'99 minus 25 - 48 to 37° 1:50 pm - 8:30 am (cold)
 Processed to 50 pl 0.001M EDTA

54 pl 10xRI salts
 459 pl ddw
 27 pl (5x27u) Nhe I (50 pl) lot 23
 20 pl + 1 pl J'99² minus (25) - (48)
 2 pl J'81 pSKB1
 2 pl J'99 (23)

37° 3:20 pm
 5:35 pm

Fri. Jan 21st

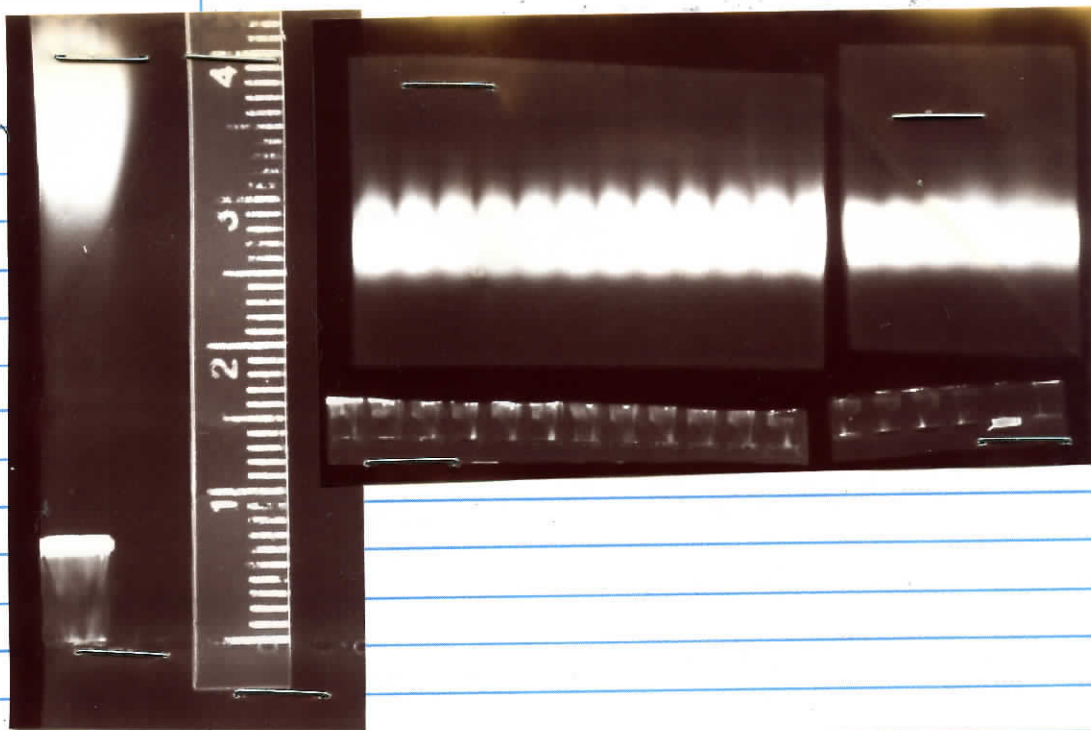
Take 40 pl 10xRI
 340 pl ddw

20 pl (10x4u) AVR II lot 29 (40 pl)

40 pl per aliquot
 each plus 2 pl J'99² minus (25) (26) (27) (28) (29) (30) (31) (32) (33) (34) (35)
 2 ret plus 8 pl J'99 mini (23)

37° 11:43 am
 1:15 pm

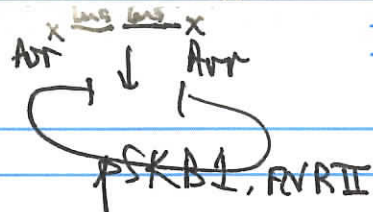
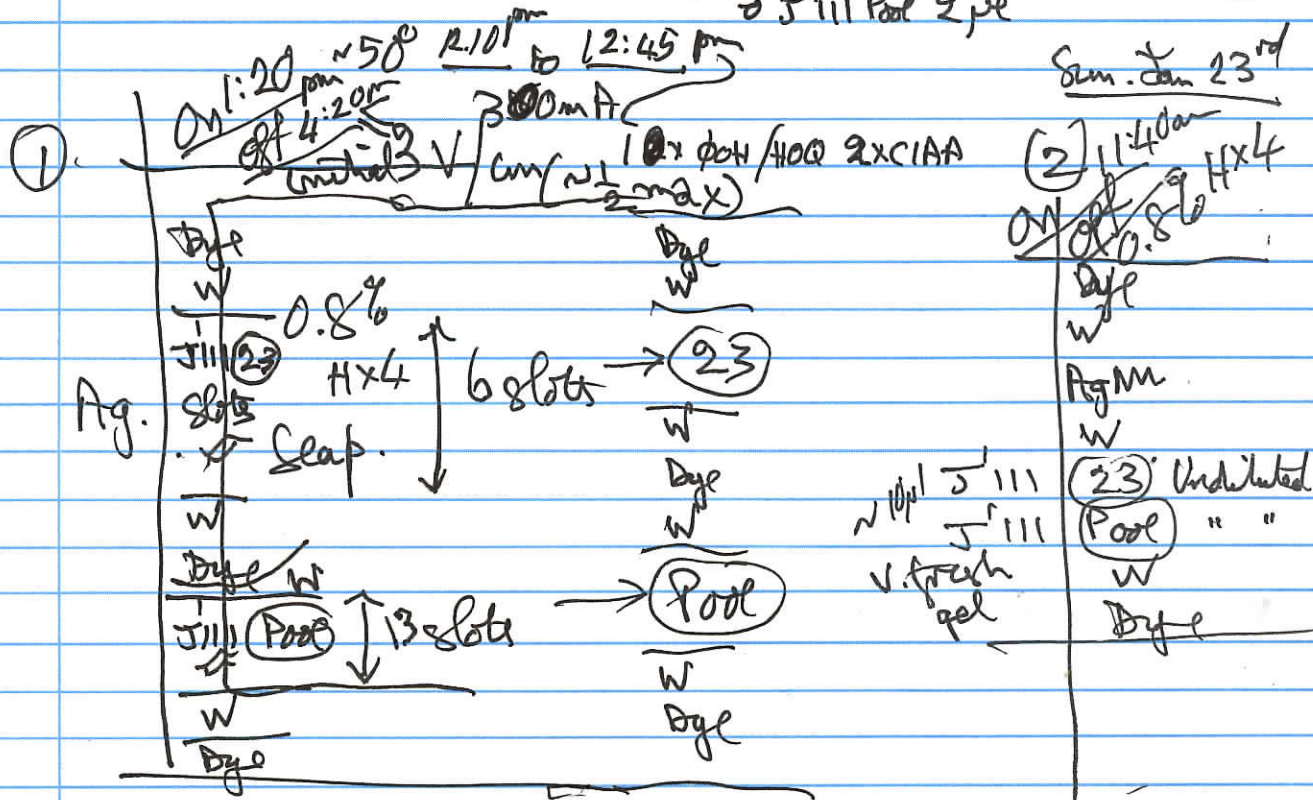
①



② Conclusion: Expected yields five products.



Sat. Jan 22nd

Infection of insulatorsJ'99 (23) AVR II from J'109 - recovered ~80 μ l as J'111 (23)J'99 2 (2) (2) (3) (3) (3) (4) (4) (4) ... ~175 μ l as J'111 PoolEach plus 1/4 vol. 5X CIP buffer & J'111 (23) 1 μ l - CIP lab stock
& J'111 Pool 2 μ lConclusions: Just inside Seaplane - a bit tricky - but cuts were good. ~~Back to~~ 1X 40H/100, 2X CIAA ~~not~~
+ 1/10 & 8M NH₄AcBack into 50 μ l

0.001M EDTA

100 μ l

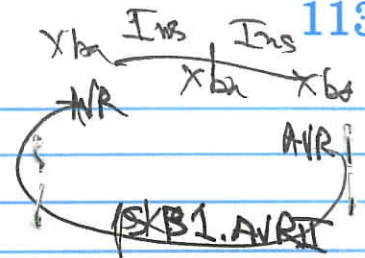
J'111 (23)

J'111 (Pool)

J'111 4 μ l J'111 (23) to
L16 whole (6 μ l) J'89Mvol. 10 μ l + 1.2 μ l 10x95+ 1.3 μ l T4 ligase lot 970
Cold 6:30 pm to 2:45 pm

Sunday Jan 23rd

Inhibitors (cont.)



Transformation

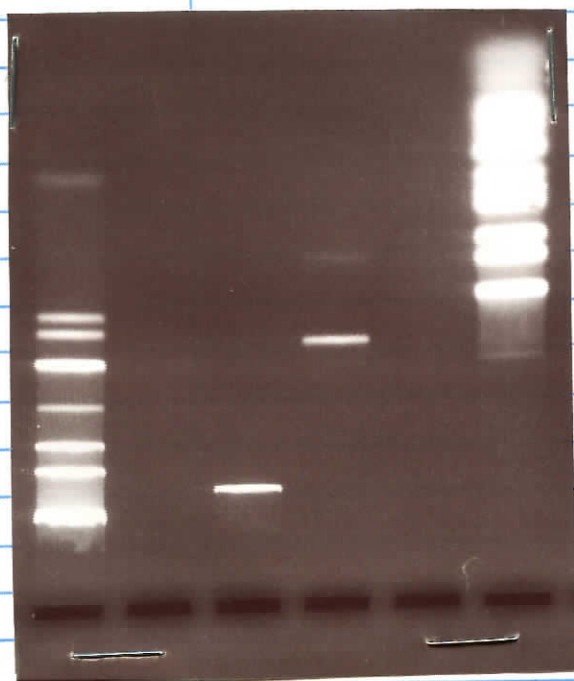
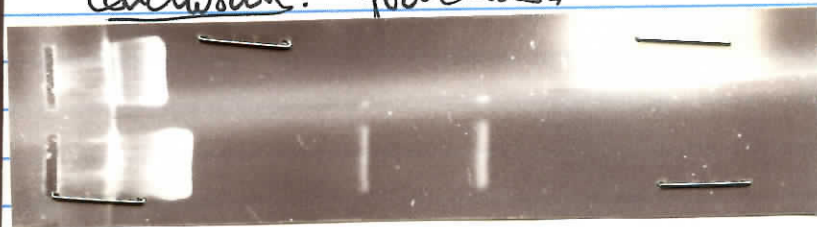
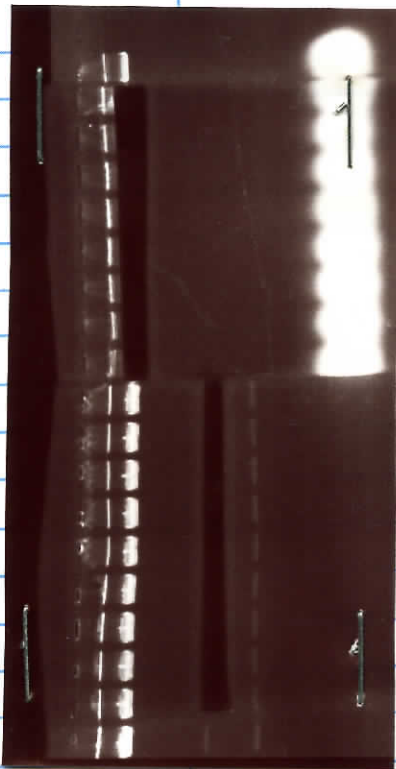
DH5α	80 µl ✓	Plated	(720) (12) (3) (0) (0)
5'111 lig.	6 µl ✓	1x Amp	R, 20, 5, 20, 5
0° 2:45 pm	3:55 pm ✓	NZy	16, 16
90 sec	42°-42.5° ✓		37° 4:00 pm
			1:20 pm

Picked 5'113 minis (1) - (24) 37° 1:45 pm

Abandoned because of
sequence results.

①

Conclusion: Good cuts.



② Conclusion: Good products
with adequate yield.
For first ligation at
this 3 to 7 ratio.

② 10:11 on	
On 9/11 11:04 am	9/11/11
0.8	0.8
Dye	
W	
PCR MM	
W	
3+12	Fragment A (1)
7+8	Fragment B (1)
	W
	PCR MM
	W
	Dye

Wed. Jan. 26th

Re-start on Insulator Inlet

Take ✓ 20 µl J'99 Red mini (11)
 ✓ 20 µl 10x RI salts
 ✓ 160 µl ddw
 ✓ 10 µl (40 v) AVR II lot 29 (4 v/µl)

J'115
(11) AVR37° 3:15^{pm} to 4:23^{pm}

Shreaked out J'99 #11 for Seigo. 37° 3:30^{pm}
 Had to repeat - poor growth - no singles.
 from bulk Turn. Jan 27th still no colonies. Seigo re-transformed

J'115 #11

CANCELLED

J'89² xba 4
still available

Take 7 µl (N10 µg) J'65 JCI3-1

25 µl 10x RI salts

218 µl ddw

Are warm to 37°

+ 1 µl (20 v) Xba I lot 30 (20 v/µl)

After 6 min

inactivated at 80°C without PBS

1/4 vol 5x CIAA buffer

+ 53 µl 1M Tris pH 8

+ 2 µl lab std. CIAA

~ 50° 4:50^{pm} to 6:05^{pm} (11)

① 12:16^{pm} to do → 1x φOH/HOQ 2x CIAA
 3V/cm 300mA (frozen)

J'115 (11)
AR CIP CIAAHX4
0.8%Dye
Water

HX4 0.8% Soap

9 slots J'115 AVR (11)
(not v. good) CIP CIAARecarved
into 50 µl

Red

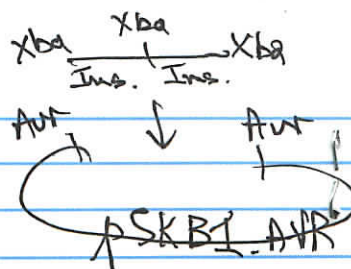
J'115
Fragment
AVR (11)W
Dye
W10 slots J'89² Xba 4
(fine)

Blue

20 µl

J'115
Fragment
Xba Deriv

Friday Jan 28th
Insulator insertion
in AVR (11)



- Take 7 µl J'111 Fragment Xba dimer
- ✓ 3 µl J'111 Fragment AVR (11)
- ✓ 1.2 µl p95 10x buffer
- ✓ 1 µl T4 ligase buffer (hot 970)

J'117
LIGN

Cold 12 noon to 11⁰⁵ am & continued

Saturday Jan 29th

Transformation

J'91 DH5α	80 µl	1x AMP NZY
J'117 lig.	4 µl	② ② ① ② ②
		R, 20, 5, 20, 5
		16, 16
0 ⁰⁰ 11 ⁰⁵ am to 11 ⁵⁰ am		
90 sec	42° - 42.3°	37° 12 noon Room temp. at 6 pm because of w. predicted

Hind III will give good check of reversed dimers & single.

pSKBI-AVR II will give a unique 6.6 kb band

AVR DIM. seq 3^{upside} bands: 2.4, 1.2 & 5.4 kb

AVR DIM REV. seq 3 bands: 3.1, 1.2 & 4.6 kb

Equivalent Monomers will miss the 1.2 kb band.

Sun Jan 30th

J'117 minis ① ② & ③ to 37° at 3 pm

Salazar

Page 1 of 3



1M. seq
↑
ood.

5:10pm
5:40pm
1.2% Hx1
RNAse
3µl/10ml
10V/cm

Mon. Jan 31st

Type
W

Salencer Primer ~~injection~~ (cont'd.)

AcMM
W

Take 90 µl 10X Eco RI salts

(1) J'117

80 µl ddw

(2) Hind

1 µl (20v) Hind III hot 52 (v/µl)

(3) J'99

W Hind

20 µl for each of J'117 minis (1), (2) & (3) and J'99 (11)
37° 3:32 to 5pm

AcMM

W

Type

ddw Template

Excellent
sequence

Seq.
144

13 µl

J'117

(1)

2 µl

J'103

Blue

100

5 µl

Submitted Feb 1st

This is for 3' end

24955

Oligo #

Date made:

LENGTH: 20

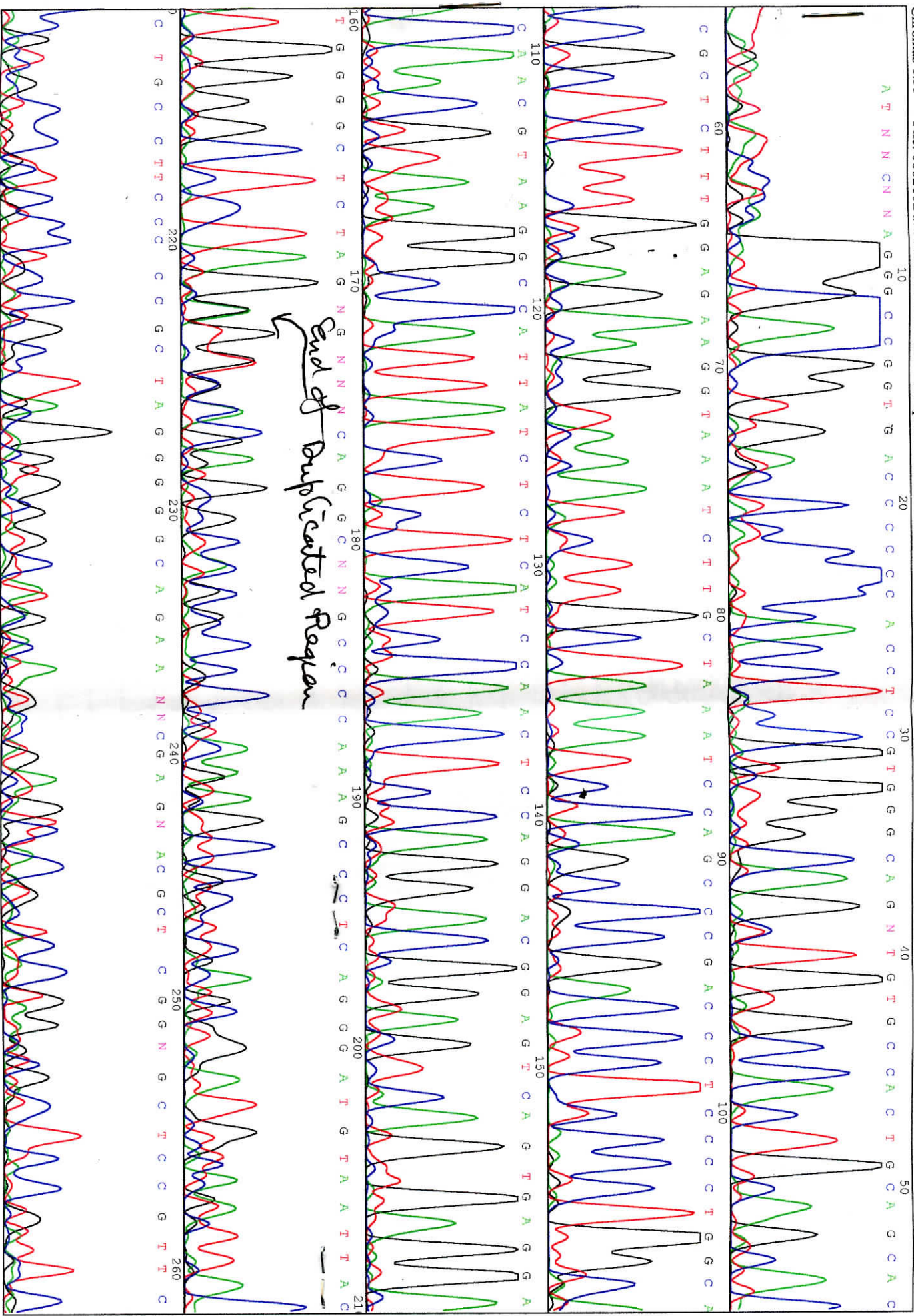
% GC: 45%

Oligo Name: J'119 Blue 1

5'	ACIA	GT	CTT	CAAT	CAAC	3'
	CITC	CIA				

J'119 Blue 1

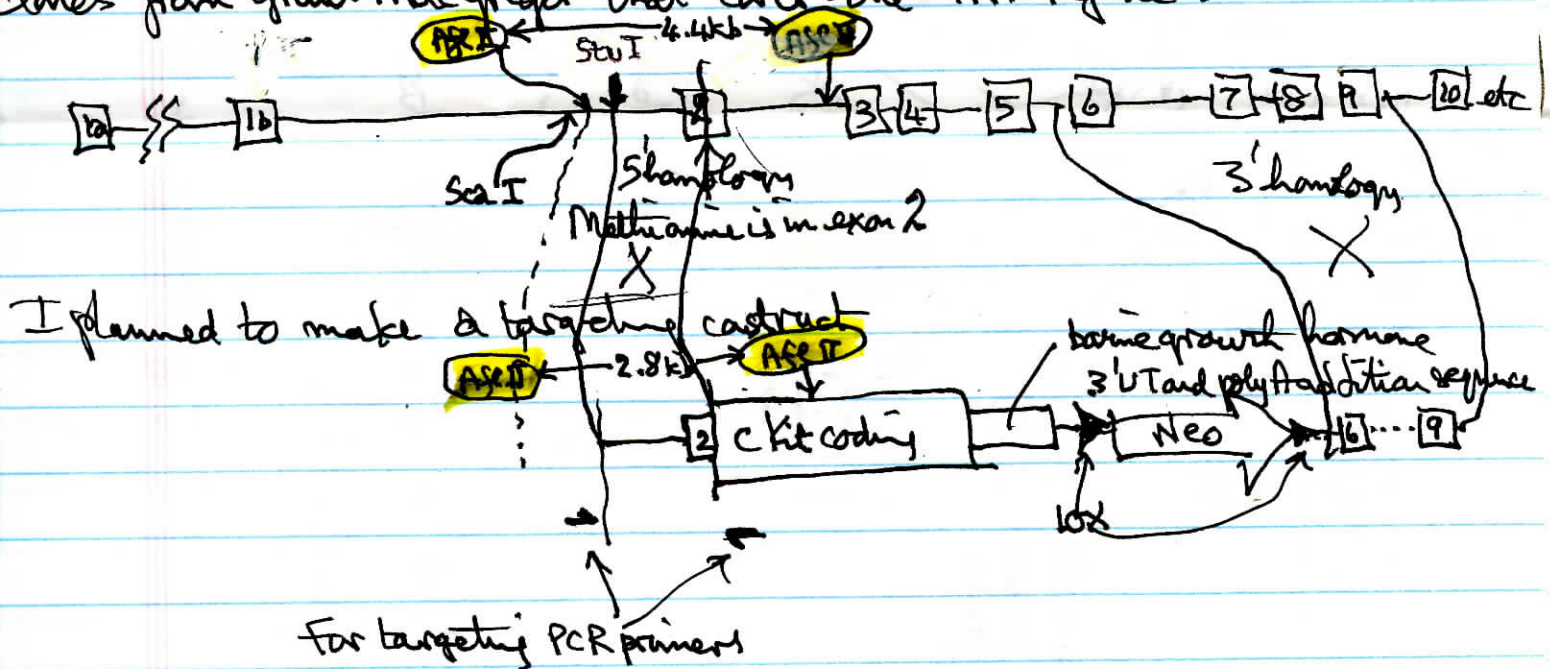
GGGTTCTGGTTGGGGTTGGGTTGGGTGGCACGGTGACCGGTACAGGGAGTCACGGTGTAGG + 7620
 CCACAGTTCTTTCATCACCTCCAGGGACGGTGACNCCNCCCTCCGTGGCAGCTGTGCC
 GGTGTCAAGAAGTAGTGGAGGTCCCTGCCACTGNGNGNGGAGGAGGCACCGTCGACACGG + 7680
 ACTGCAGCACCGCTCTTTGGAGAAGGTAAATCTTGCTAAATCCAGCCCGACCCCTCCCCTG
 TGACGTCGTGGCGAGAAACCTCTTCCATTAGAACGATTTAGGTCGGGCTGGGAGGGGAC + 7740
 GCACAACGTAAGGCCATTATCTCTCATCCAATCCAGGACGGAGTCAGTGAGAATATTxx
 CGTGTGCAATCCGGTAATAGAGAGTAGGTTGAGGTCCTGCCTCAGTCACTCTTATAAxx + 7800
 xxTCTAGgttCCGGCGTAGGCGCGCGGGCGGGGCGGGGCGGGGCGGGGCGGGGCGGTGGC
 xxAGATCcaaggCCGCATCCGCGCGCCCGCCCCGCCCCCGCCCCGACGCCCCGCACCG + 7860



Tissue Non-Specific Alkaline Phosphatase (TNAP) replacement with c Kit, the receptor for Stem Cell Factor

Background TNAP has been known for many years to be a marker for cells that eventually become germ cells. My thoughts, since heterozygotes for TNAP gene disruption are essentially normal, to replace the TNAP coding region by c Kit so that the receptor would be made earlier in development than usual, and might enable ES cells with this TNAP c Kit to outcompete wild-type cells in populating the germs.

I got c Kit cDNA from Alan Bernstein, and four clones from Grant MacGregor that cover the TNAP gene:—



The construction went as planned & also included a ~~positive~~ positive control. Details on next page.

Tues. April 18th

INAP ckit

Review prior to transfer to Bud Koller.

PCR use NM I'33 NM Blue
and OS G'117 TK Red as PCR primer

Use Afl II for Southern 2.8 kb target
4.4 kb endogenous
with G'47 XKT frag. - a 1.1 kb Xba/Kas from G'29T
luciferase

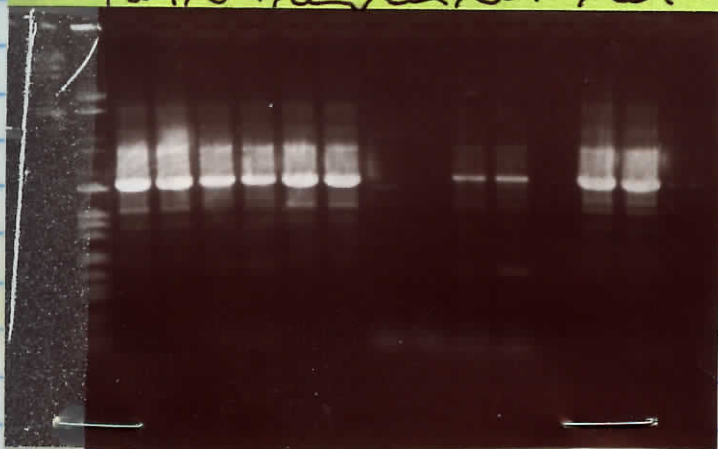
Thurs. May 4th

Sylvie grew up more targeting plasmids from
fresh transformation using G'125 (T1) minus &
carried out PCR checks with it & with control plasmid & control cell
using: Truncated F'162 Blue + G'117 TK Red G'125 (C23) (185) diff
Targeting I'33 Blue NM

All were + with F'162 as expected; T1 was - with I'33 Blue; C23 & 185
were + with I'33 Blue. ∴ correct.

Final check is a G'128 repeat digest with Asc I + Kas I

Control plasmid Control plasmid
G'125 1BE G'125 G'125 + G'125
TI ES C23 TI ES C23
24 Feb 92



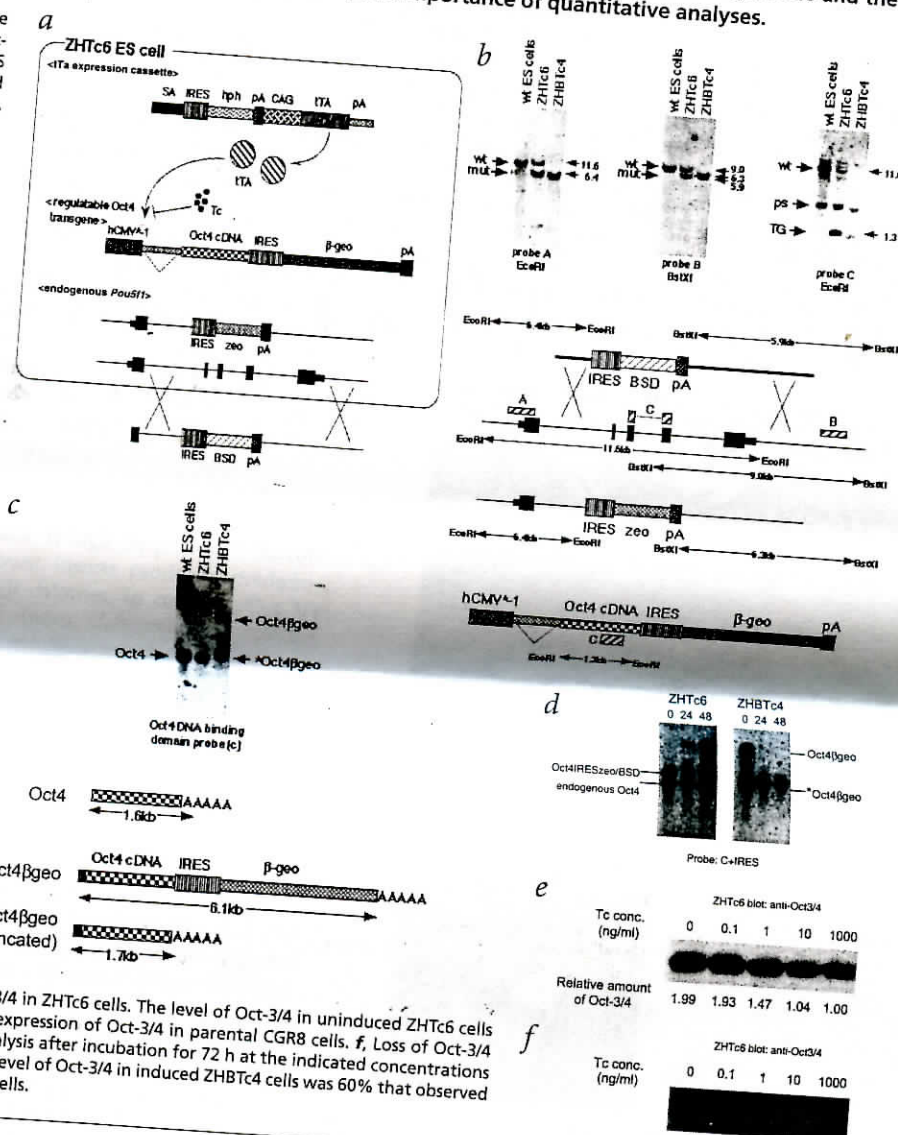
Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells

* Hitoshi Niwa^{1,2}, Jun-ichi Miyazaki² & Austin G. Smith¹

Cell fate during development is defined by transcription factors that act as molecular switches to activate or repress specific gene expression programmes. The POU transcription factor Oct-3/4 (encoded by *Pou5f1*) is a candidate regulator in pluripotent and germline cells¹⁻⁴ and is essential for the initial formation of a pluripotent founder cell population in the mammalian embryo⁵. Here we use conditional expression and repression in embryonic stem (ES) cells to determine requirements for Oct-3/4 in the maintenance of developmental potency. Although transcriptional determination has usually been considered as a binary on-off control system, we found that the

precise level of Oct-3/4 governs three distinct fates of ES cells. A less than twofold increase in expression causes differentiation into primitive endoderm and mesoderm. In contrast, repression of Oct-3/4 induces loss of pluripotency and dedifferentiation to trophoblast. Thus a critical amount of Oct-3/4 is required to sustain stem-cell self-renewal, and up- or downregulation induce divergent developmental programmes. Our findings establish a role for Oct-3/4 as a master regulator of pluripotency that controls lineage commitment and illustrate the sophistication of critical transcriptional regulators and the consequent importance of quantitative analyses.

Fig. 1 Generation of ES cells with variable Oct-3/4 expression. a, ES cell lines were generated by electroporation of 129/Ola ES cell embryos with a 1.5 kb Oct-3/4 promoter construct. b, ES cell lines were screened for Oct-3/4 expression by RT-PCR. c, ES cell lines were screened for Oct-3/4 expression by immunofluorescence. d, ES cell lines were screened for Oct-3/4 expression by Western blot analysis.



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Mon. April 24th

On the failure to get recombinant
C57BL/6 ES cell lines

Hyoung-Suk Kim succeeded in getting gene disruption & gene duplication in ^{claw #1} of the C57BL/6 ES cell line ^{from Switzerland}, but it has ^{an unstable karyotype} and does not ^{go from line}. Tomoko Hatake has had no success with ^{claw #2} in getting homologous recombination, although it gives better chimeras (not yet germ line). The attached paper by Niwa et al. (Nature Genetics 24, 372, 2000) suggests that Oct-3/4 could be affecting both h.r. and germline trans. But, in any case, the 2 cell lines suggest that using the ABI 7700 and/or a microarray we may be able to find out how to change these two factors — perhaps leading to a transient expression platform to ↑ h.r. without later ↓ germline t. Kim, Tomoko & Laura Reid will investigate this possibility.

3-11-11
This invention provides a general method for isolating hematopoietic stem cells or other cells in which a faulty (mutant) gene has been corrected by homologous recombination (gene targeting).

✓
No PHS
Preamble. It is generally accepted that the direct correction of a faulty gene (such as the mutant form of the beta globin gene which causes sickle cell anemia) would provide an extremely useful means of treating this and other genetic diseases of a similar nature provided:

- NO PHS
i) that the correction could be achieved in stem cells capable of populating the bone marrow. [Such cells are usually called hematopoietic stem cells (HSC)].
ii) that the procedure could be executed in a manner allowing HSC in which the gene correction had been achieved to be recovered from the starting bone marrow and separated from cells in which the gene was uncorrected or in which undesirable secondary effects had occurred, such as incorporation of the correcting DNA sequences into parts of the genome other than the target (faulty) gene.

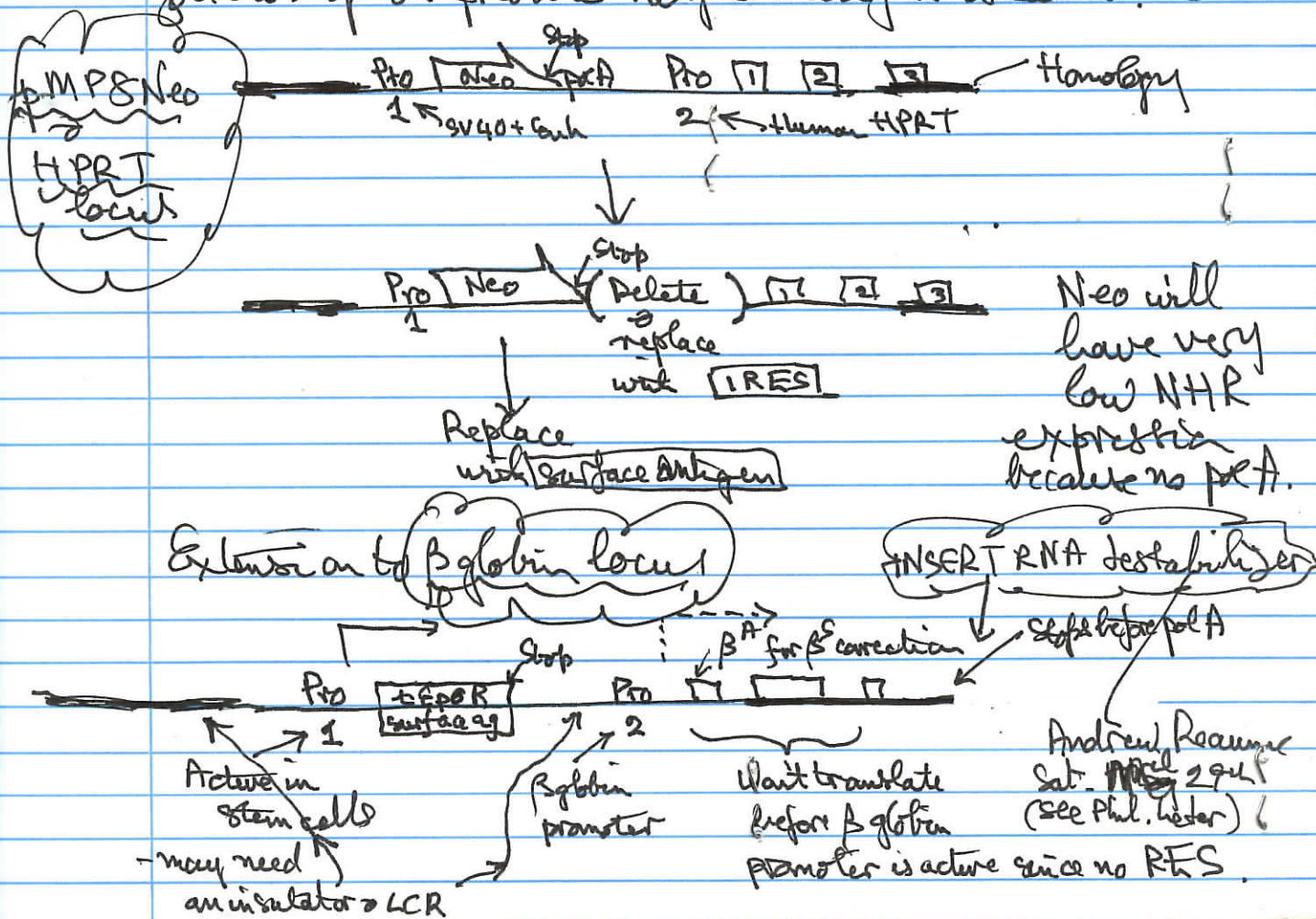
Prior work has established that homologous recombination with a correcting DNA introduced into mutant cells can correct a faulty gene, but the

For. April 28th

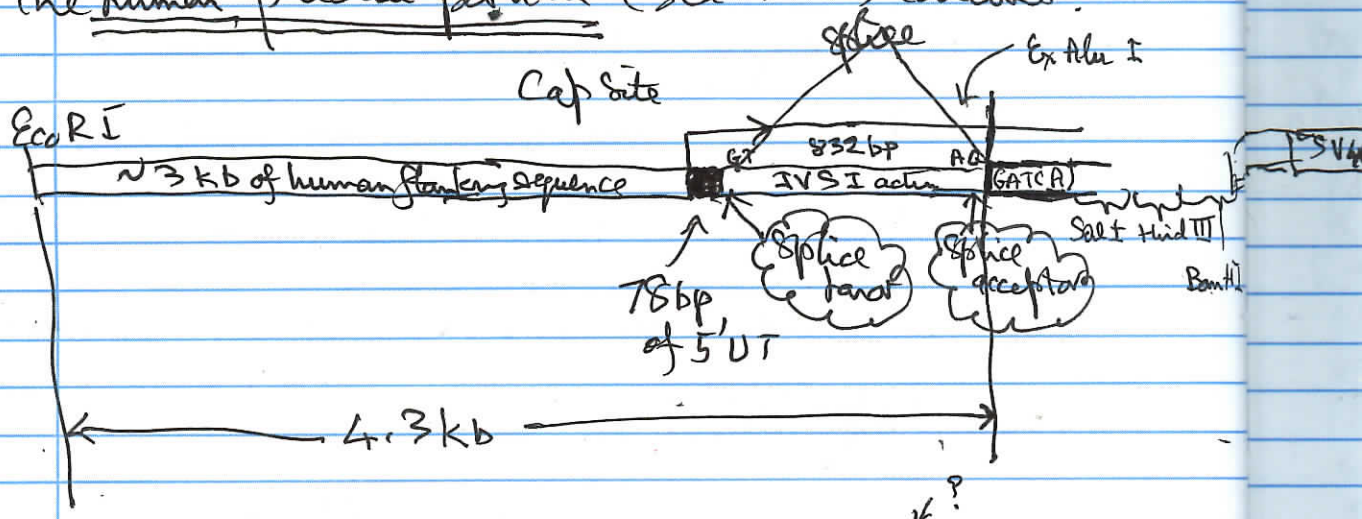
But see Paulink, Eaves & Humphries for prior use of same idea

On surface antigen and multiple promoters

In carrying out homologous recombination with pMP8Neo and hematopoietic progenitor cells, we have been using HAT selection which required ≥ 14 days $\rightarrow$ cell toxicity. Need to use that w/ hematopoietic stem cells if possible without HAT selection $\rightarrow$ growth. A possible solution that follows up on previous surface antigen idea is: -



The human β actin portion (see TT 66) contains: -



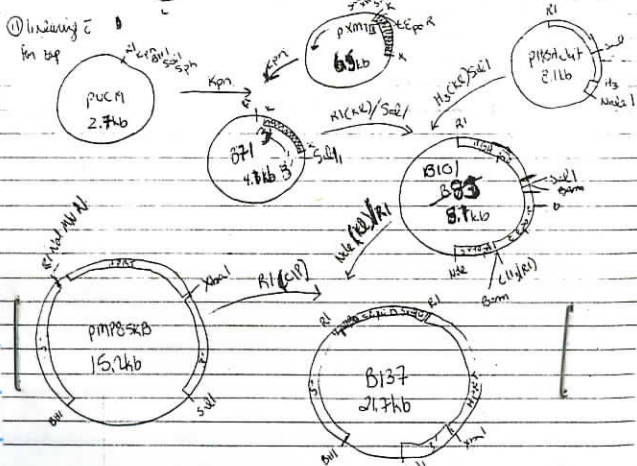
The tEpoR⁻ is truncated at -91 (c.r. human mutant at -41).

56

see map p. 56

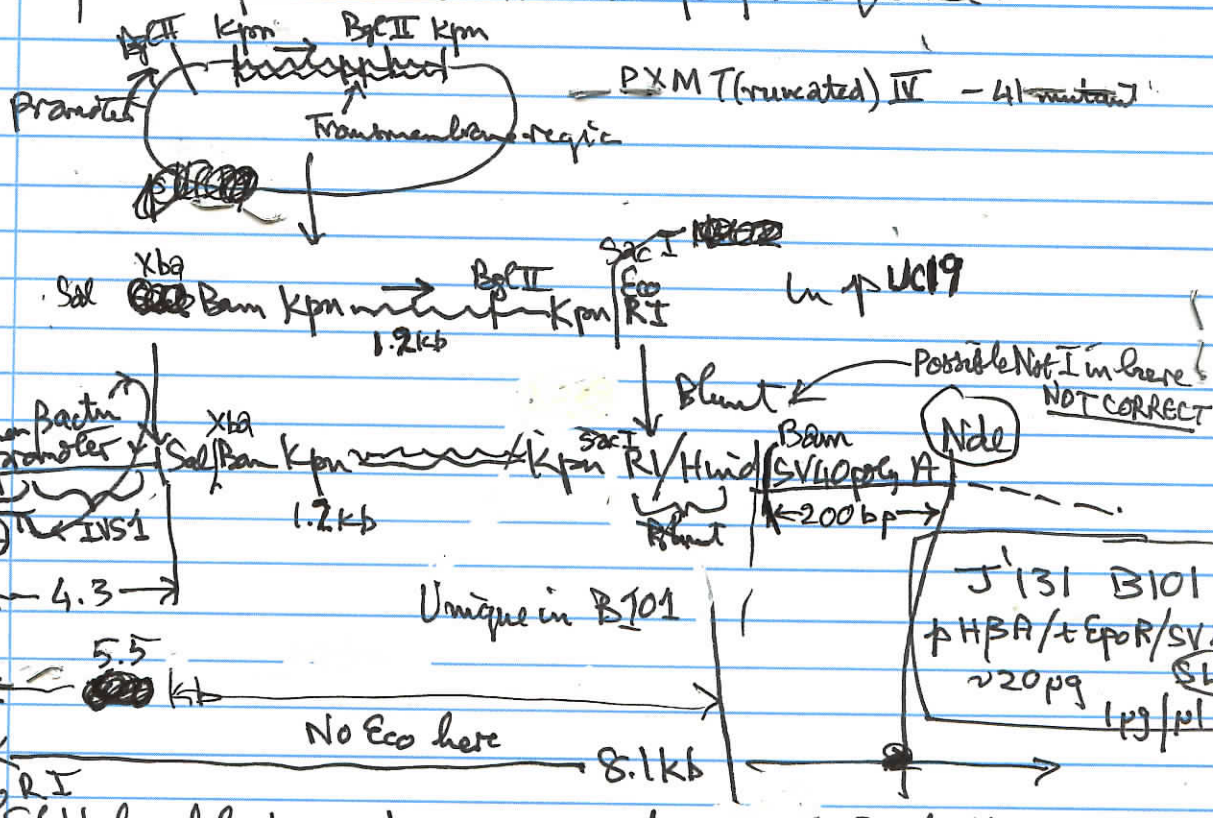
CLONING STRATEGIES for tEpoR

1. pUC19 Kpn (CMT) + CMT Kpn → BSS BSS
2. Verification: BamHI, Bss, XmnI, Sma, Pst
3. grow up right one
4. cut BSS c. EcoRI, Klenow, 0.1% CMT, then SalI
5. electroporate 1.4 kb frag. d. EtOH, p.p. d. neomycin
6. Klenow cut pUC19 hit c. Itz, Klenow, then SalI d. electroporate 1.8 kb frag.
7. ligate tEpoR frag. R1(c2)/Sal to pUC19 0.8 frag. Itz d. pick colonies for minis → BSS BSS BSS
8. digest to verification d. convert (E. mant) c. 0.1% CMT
9. again grow up plasmid (BSS) d. cut c. Nde → Klenow → then R1 d. electroporate frag. non-act. stop
10. cut pMP85B c. R1 d. ligate to 0.8 frag. BSS
11. pick colonies for minis d. convert by digest



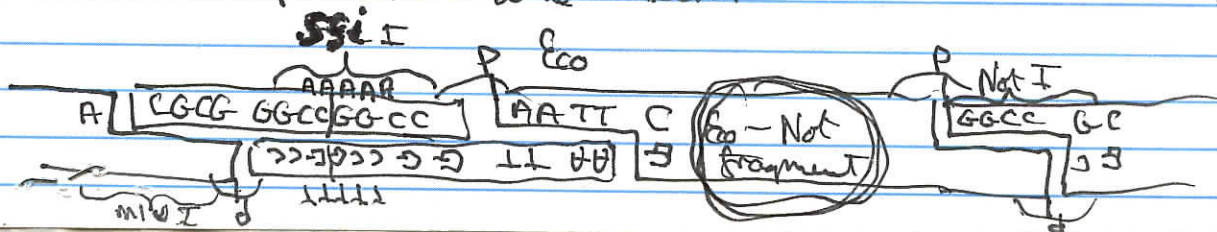
mouse

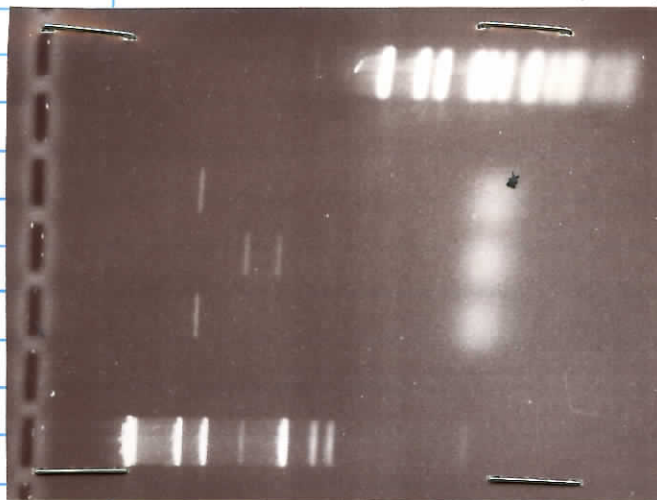
Suzanne started with a Epo cDNA plasmid $\rightarrow$ excised coding region with Epo I , resealed in pUC $\rightarrow$ cut out again with Eco RI Kpn $\rightarrow$ Kpn Bam Sal $\rightarrow$ inserted into a ckt plasmid driven by human Bactⁿ promoter with SV40 poly A sequence.



Should be able to cut out reg. fragment w/ NotI and EcoRI
to put into pSKB ARII (without negative regulatory element).
vary the NotI cloning site. Will need to check linearization
with PmeI or AseI ~ 6 kb

- i) Check Eco, Not and Eco+Not on BIO1 & prepare EcoRI — NotI
- ii) Cut pSKB. Aor II with MluI and Not I & ? purify.
- iii) Consider ligation with some linkers: —





Conclusion

Sample Eco RI & a
sample Not I site, but
size of product & conc. is
very low.
Total length is ~8kb - which
is expected length (8.1kb).
Not/Eco frag. are
3.5 & 4.5

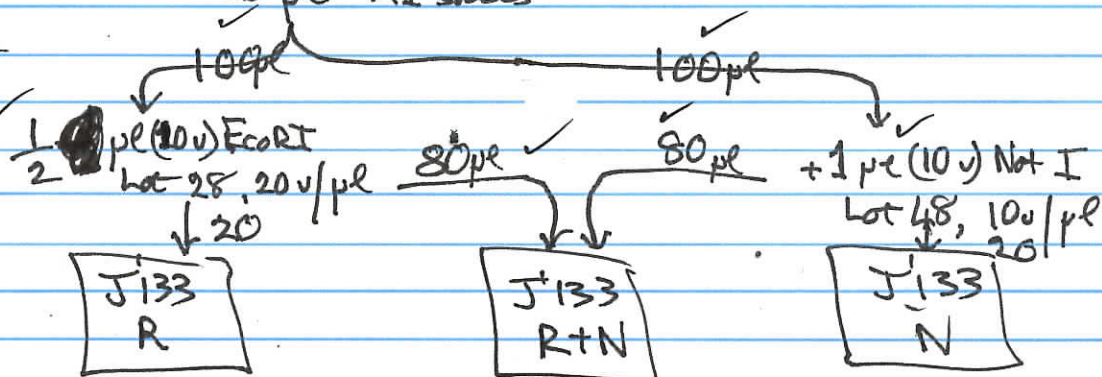
? PVC 2.7 ? 4.3 from Bactin 30 Can

MEL EPO Receptor cDNA Sequence

11 28 40 52 76
 tgaagcttccct gaagctaggg ctgcatc ATG GAC AAA CTC AGG GTG CCC CTC TGG CCT CGG GTA GGC CCC CTC TGT CTC CTA CTT GCT GGG GCA GCC TGG
 M D K L R V P L W P R V G P L C L L L A G A A W
 109 124 148 172
 GCA CCT TCA CCC AGC CTC CCG GAC CCC AAG TTT GAG AGC AAA GCG GCC CTG CTG GCA TCC CGG GGC TCC GAA GAA CTT CTG TGC TTC ACC CAA CGC
 A P S P S L P D P K F E S K A A L L A S R G S E E L L C F T Q R
 196 220 244 268
 TTG GAA GAC TTG GTG TGT TTC TGG GAG GAA GCG GCG AGC TCC GGG ATG GAC TTC AAC TAC AGC TTC TCA TAC CAG CTC GAG GGT GAG TCA CGA AAG
 L E D L V C F W E E A A S S G M D F N Y S F S Y Q L E G E S R K
 292 316 340 368
 TCA TGT AGC CTG CAC CAG GCT CCC ACC GTC CGC GGC TCC GTG CGT TTC TGG TGT TCA CTG CCA ACA GCG GAC ACA TCG AGT TTT GTG CCG CTG GAG
 S C S L H Q A P T V R G S V R F W C S L P T A D T S S F V P L E
 388 412 436 460
 CTG CAG GTG ACG GAG GCG TCC GGT TCT CCT CGC TAT CAC CGC ATC ATC CAT ATC AAT GAA GTA GTG CTC CTG GAC GCC CCC GCG GGG CTG CTG GCG
 L Q V T E A S G S P R Y H R I I H I N E V V L L D A P A G L L A
 484 508 532 556
 CGC CGG GCA GAA GAG GGC AGC CAC GTG GTG CTG CGC TGG CTG CCA CCT CCT GGA GCA CCT ATG ACC ACC CAC ATC CGA TAT GAA GTG GAC GTG TCG
 R R A E E G S H V V L R W L P P P G A P M T T H I R Y E V D V S
 580 604 628 652
 GCA GGC AAC CGG GCA GGA GGG ACA CAA AGG GTG GAG GTC CTG GAA GGC CGC ACT GAG TGT GTT CTG AGC AAC CTG CGG GGC GGG ACG CGC TAC ACC
 A G N R A G G T Q R V E V L E G R R T E C V L S N L R G G T R Y T
 676 700 724 748
 TTC GCT GTT CGA GCG CGC ATG GCC GAG CCG AGC TTC AGC GGA TTC TGG AGT GCC TGG TCT GAG CCC GCG TCA CTA CTG ACC GCT AGC GAC CTG GAC
 F A V R A R M A E P S F S G F W S A W S E P A S L L T A S D L D
 772 796 820 844
 CCT CTC ATC TTG ACG CTG TCT CTC ATT CTG GTC CTC ATC TCG CTG TTG CTG ACG GTT CTG GCC CTG CTG TCC CAC CGC CGG ACT CTG CAG CAG AAG
 P L I L T L S L I L V L I S L L L T V L A L L L S H R R T L Q Q K
 868 892 916 940
 ATC TGG CCT GGC ATC CCA AGC CCA GAG AGC GAG TTT GAG GGT CTC TTC ACC ACC CAC AAG GGT AAC TTC CAG CTG TGG CTG CTG CAG CGT GAT GGT
 I W P G I P S P E S E F E G L F T T H K G N F Q L W L L Q R D G
 964 988 1012 1036
 TGT CTG TGG TGG AGC CCG GGC AGC TCC TTC CCT GAG GAT CCA CCT GCC CAC CTA GAG GTC CTC TCA GAG CCA CGC TGG GCA GTG ACT CAG GCT GGG
 C L W W S P G S S F P E D P P A H L E V L S E P R W A V T Q A G
 1060 1084 1108 1132
 GAC CCA GGG GCA GAT GAT GAG GGG CCC TTA CTG GAG CCG GTG GGC AGT GAG CAT GCC CAG GAC ACC TAC TTG GTA TTG GAT AAG TGG TTG CTG CCC
 D P G A D D E G P L L E P V G S E H A Q G D T Y L V L D K W L L P
 1156 1180 1204 1228
 CGG ACC CCA TGC AGT GAG AAC CTC TCA GGG CCT GGG GGC AGT GTG GAC CCT GTG ACT ATG GAT CAA GCT TCA GAA ACA TCT TCC TGC CCG TCT GAC
 R T P C S E N L S G P G G S V D P V T M D E A S E T S S C P S D
 1252 1276 1300 1324
 TTG GCC TCA AAG CCC AGG CCA GAG GGC ACC TCA CCT TCC AGC TTT GAG TAC ACC ATC CTG GAC CCC AGC TCT CAG CTC CTG TGC CCT CGG GCA CTG
 L A S K P R P E G G T S P S F E Y T I L D P S S Q L L C P R A L
 1348 1372 1396 1420
 CCT CCC GAG CTA CCT CCC ACT CCA CCT CAC TTG AAG TAC CTA TAC CTT GTG GTG TCC GAT TCT GGC ATC TCA ACA GAT TAC AGT TCG GGG GGC TCT
 P P E L P P T P P H L K Y L Y L V V S D S G I S T D Y S S G G S
 1444 1468 1492 1516
 CAG GGA GTC CAC GGG GAC TCA TCT GAT GGC CCC TAC TCC CAC CCC TAT GAG AAC AGC CTT GTC CCA GAC TCA GAG CCT CTG CAT CCC GGC TAT GTG
 Q G V H G D S S D G P Y S H P Y E N S L V P D S E P L H P G Y G
 1540 1562 1582 1602 1622 1642
 GCC TGC TCC TAG gactccagcc tacaacgtct tgaacgggat tgggtgaagcc ata cttaaag tcagagctga ccttgccct ctgagcagga agagacagcc ttgcaatgtt
 A C S
 1662 1682 1702 1722
 aagattaaga gttatctgtc tgtatataga aatatatata tatatcgatt ttctacc tt gaaaaaaaaa aaaaaaaaaa aaaaaaaaaa

Take $\checkmark$ 3 μ l (3 μ g) J131 B101 (1 μ g/ μ l)
 $\checkmark$ 177 μ l ddw
 $\checkmark$ 20 μ l RT salts

Dye
W.
ACR
W
R
RTN
W
W
AG
W
~~Dye~~



37° 7pm - 8:10pm (frozen)

Fri. May 12th

Careful check makes it likely that there is a Not I site between BstI primer and the $\pm$ EpoR fragment, but pUC19 doesn't have Not I in linker. Best bet is to grow up more B101 $\rightarrow$ sequence in blue direction from within $\pm$ EpoR cDNA $\rightarrow$ find out what is happening.

Oligo# _____
Date made: _____

J'133 Bho FPR

5' Oligo Name: _____

TIGG	AACC	CITG	TGIA	CITA
TGGG	ATT			

LENGTH: 20
% G+C: 50%

Oligo # _____
Date made: _____

5' Oligo Name: J'133 Blue 2

C	T	C
A	G	G
G	C	C
T	G	G
G	G	G

LENGTH: 17
% G-C: 76%

500

1. D

1.0

1.0

2.1 2.1 2.1 2.1

3.1

27

27

2.10

3.5

4.5

4.6

5.4

6.0

5.4

71

13

Eco Eco Eco Eco Nde Hind Eco Bam Eco Eco
Nde Nde Nde Nde Hind Bam Nde

CAP SITE EVOLUTION OF THE FUNCTIONAL HUMAN β -ACTIN GENE

777

Not I

[illegible]

① Conclusion from map is that there are 3 Bam HI sites, the 1st is in the β actin gene. Best bet is B5m I (see J137 & 139)

Sat. May 13th

Mapping B101

Ag-MMM

W

EcoR + Nde + Hind

EcoR + Nde + Bam

EcoR + Nde + Not

EcoR + Nde

Nde

~~205~~ 205 ~~100~~ 100 ~~pe~~ pe ~~200~~ 200

Take ~~205~~ 205 ~~100~~ 100 ~~pe~~ pe (205 ~~100~~ 100) 5' 131 B101

25 ~~100~~ 100 ~~pe~~ pe 10x RI salt

205 ~~100~~ 100 ~~pe~~ pe 200

~~205~~ 205 ~~100~~ 100 ~~pe~~ pe

~~205~~ 205 ~~100~~ 100 ~~pe~~ pe

~~205~~ 205 ~~100~~ 100 ~~pe~~ pe

~~205~~ 205 ~~100~~ 100 ~~pe~~ pe

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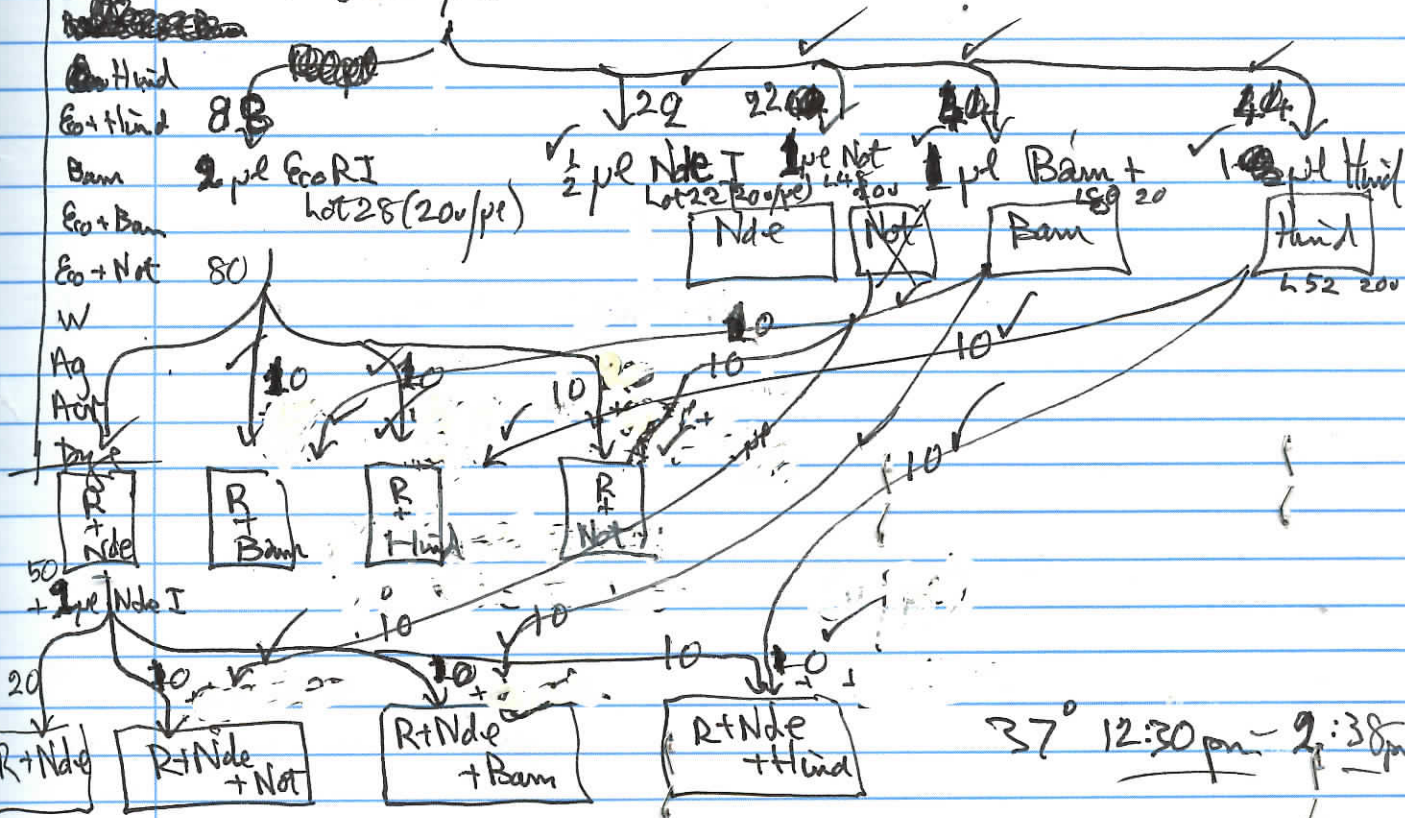
0.5 ~~100~~ 100 ~~pe~~ pe 200

0.5 ~~100~~ 100 ~~pe~~ pe 200

0.5 ~~100~~ 100 ~~pe~~ pe 200

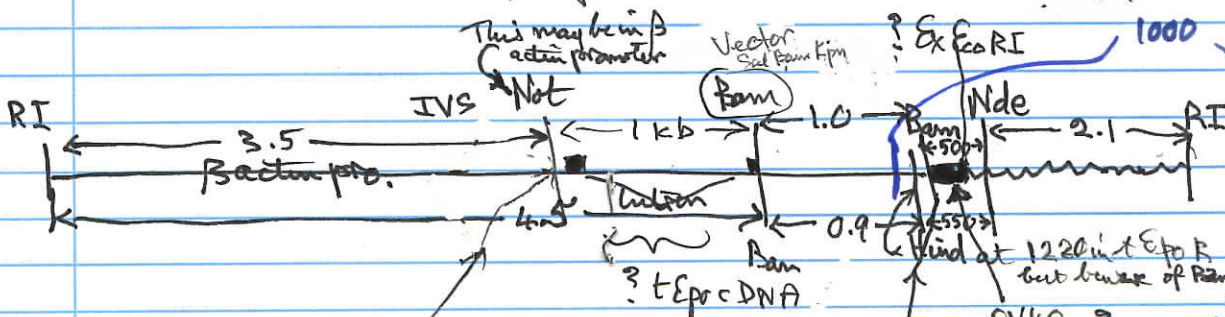
0.5 ~~100~~ 100 ~~pe~~ pe 200

0.5 ~~100~~ 100 ~~pe~~ pe 200



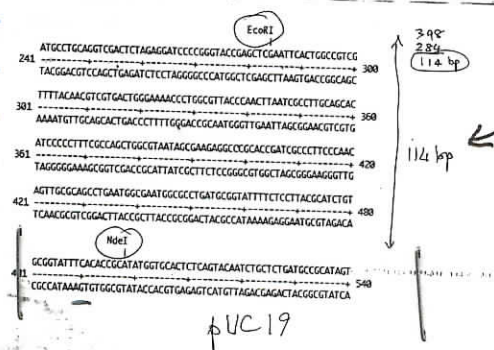
37° 12:30 pm - 2:38 pm

Conclusion: map agreed O.S. & N.M.

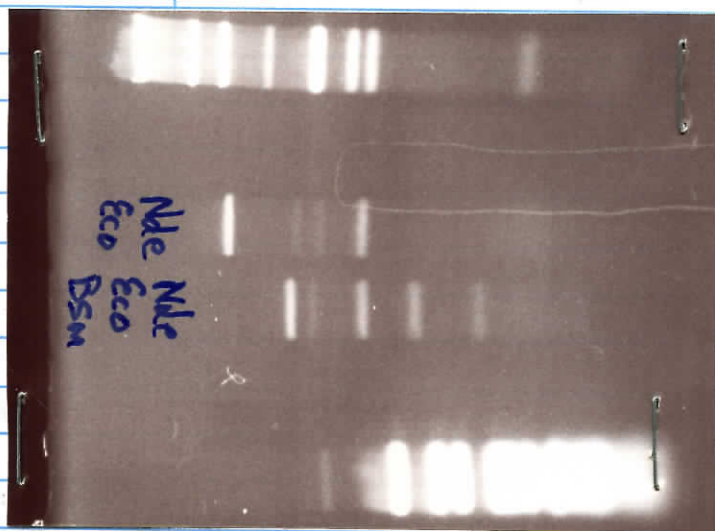


There is a NotI at -50 from T and 1 kb up stream of fragment end.

? Bam between SV40 polyA and TER



← No NotI cut here. EcoRI & NdeI is included in NdeI



⑦ Conclusion. Two BSM I sites - one is correct but other is probably in β -actin promoter. Still it is earlier than the BSM situation of spacing is better.

Sun May 14th

B101 regrowth

If

(old tube - label gone) } D H5X J'137 80 μ l
J'13 B101 1 μ l

0° 11:57 am - 2:20 pm

42° 40 sec

Plated NZY R, 20, 5, 20/16, 1/16

lamp

Many

37° 1:30 pm - to 9 am

but it had shut down

To confirm for grow up & sequencing

On 5:05 pm
off 6:10 pm
0.8% Hx4 RNAse 3/10

Cutting of poly A from RI - Nde of B101

Agg (1)

Agg

W

Eco+Nde

W

Agg

W

Agg

Bam HI looks very hard (3 sites). Bsm I is possible. fudging from SV40 late poly A region. Site isn't in tEpoR or P3864 - sequenced part of Bacteriophage (last 1 kb).

Eco+Nde + Bsm I Take 20 μ l of J'135 EcoR + Nde left over + 1 μ l (50)

Bsm I hot 10 (5 μ l) 37° at 3:53 pm to 4:55 pm

& 20 μ l of rest as control

Note it had been at r.t. overnight.

28 40 52 76
 atc ATG GAC AAA CTC AGG GTG CCC CTC TGG CCT CGG GTA GGC CCC CTC TGT CTC
 M D K L R V P L W P R V G P L C L
 124 148 172
 JAC CCC AAG TTT GAG AGC AAA GCG GCC CTG CTG GCA TCC CGG GGC TCC GAA GAA
 J P K F E S K A A L L A S R G S E E

CGGCGAAGCC GGTGAGTGAG CGGCGCGGGG ^{B actin} GCAATCGCGT GCGCCGTTCC GAAAGTIGCC TTT
 1 ACCGCCGA GACCGCGTCC GCCCGCGAG CACAGAGCCT CGCCTTTGCC GATCCGCCGC CCG
 CCTTCGCCCG TGCAGAGCCG CCGTCTGGGC CGCAGCGGGG GGC GCATGGG GGGGGAACCG GAC
 CCAGTTCGGA GGC GCGAGGC CGCGCTCGGG AGGCGCGCTC CGGGGGTGCC GCTCTCGGGG CGC
 GGGCGCGGGC TAGCCCCCGC CAGGCCCGGT GGGGGCTGGG GCGCCATGCG CGTCCGCGCT GG
 GGGACTCAAG GCGCTAATTG CGGCTGCGTT CTGGGGCCCC GGGTGCCGCG GCCQGGGCGG GG
 CTTECTGCCC GAGCCGCGGG CCGCCCGAGG GTGTGGCCGC TCGTGCGCG CGCGCGACCC GG

support sequences
 ✓
 ✓

Mon May 15th

Some more sequence checks on B101

J'139 Red Epo is Red direction from v8 aas into tEpoR.

J'139 Red Act is Red direction from conserved sequence in actin ~~promoter~~ IVS1.

		Oligo Name: J'139 Red Epo					3'
#		G C C	T A C	C C C	A G G	C C A	
Index:							
TH:	17	G A T					
C:	71%						

		Oligo Name: J'139 Red Act					3'
#		G A C	G C G	G T C	T C G	G C G	
Index:							
TH:	17	G T T					
C:	71%						

S.H. seq sequences: -

✓ 146. seq J'139 Red Act with her B101 growth

B101 EPOR
SHd50
0.65µg/µl

✓ 147. seq J'139 Red Epo

✓ 148. seq J'139 Blue Epo R

✓ 149. seq J'139 Blue 2

150. seq

J'142 Red Actin

Analysis

with B101 growth

J'140

Perfect match to human Bacter genome
SMART Published human Bacter

541 GGGCGCGGGGGCCACGGCGCGCGCCCCAGCCCCGGGCCCAGCACCCCAAGGCGGCCAA

CCCGCGCCCCGGTGC CGCGCGCGGGGGTTCGGGGGGCCGGGTCGTGGGGGTTCCGCGCGGTT 600

EarI

CGCCAACTCCTCCTCTCTTCTCAATCTGCTCTGCTCTTTTTTTTCGCA
601 + + + + +
CGGTCTTTTGAGAGGGAGGAGAAGGAGTAGAGCGAGCGAGAAAAAAGCGT 660

AAAGGAGGGGAGAGGGGGTAAAAAATGCTGCACTGTGCGGCGAAGCCGGTGAGTGAGCG
TTTCCTCCCTCTCCCCATTTTTACAGCTGACACGCGGCTTCGGGCACTCACTGCG


BsiEI
EagI
NotI
XhoI

GCGCGGGGCCAATCAGCGTGC GCGGTCCGAAAGTTGCCTTTATGGCTCGAGCGGCCG

721 ----- 780

BsaHI
 NarI
 BglI

GGCGGGCGCCTATAAAACCCAGCGCGCGAGCGGGCCTCC
 CCGCGCGCGGATATTTGGGTCGCGCGCTGCCCGGAGG

781 -----> 819

(J139 Pad Act)
 Primer was for conserved sequence
 actin intron

Bsu36I AscI MscI (147.5bp)
 651 CCCCTGGCGCGCTAAGGACTCGGCGCGCGGAAGTGGCCAGGCGGGGGCCATTGGC
 GGGGACCGCGCGGATTCTCGAGCGCGCGGCTTACCGTCCCGCCCGCTGAAGCCG
 Human *Salmonella* *met*
 Bacter *met*
 HincII AccI BamHI
 SalI XbaI BstYI KpnI
 721 TCACAGCGCGCCGGCTATTCTCGGCGATCAGTCGACTTAGAGGATCCCCGGGTACGC
 AGTGTGCGCGGGGCCATAAGAGCGTCTTAGTCAGCTGAGATCTCTAGGGGGCCATGGC
 781 CTAGGGCTGCATCATGTGACAAACNAGGGTGCCCC
 815
 GATCCGACGATGACTCTGTTTNGTCCACGGGG
 tEpoR

Comparison of 149 seq **7148**
with EboR and SV40 May 27, 2000

Eco57I HindIII BaeI
 GTAGTGGCCCCCTGTGACTATGGATGAAGCTTCAGAAACATCTTCTGCCCGTCTGACTAA
 1 CATCACCGGGGCACTGATACTACTTCGAAGTCTTTGTAGAAGGACGGGCAGACTGATT
 V V A P V T M D E A S E T S S C P S D
 KpnI BlnI ScaI BlnHI BstYI
 BlnI
 61 GTGACCGAGCTCGAATTAGCTTCGAGGATCCAGACATGATAAGATACATTGATGAGTTT
 CCATGGCTCGAGCTTAATCGAAGCTTCTCTAGGCTGTGACTATTCTATGTAACTACTCAA
 120 poly
 G T E L E L A S R D P M I R Y I D E F
 BsmI BtsI 2460
 ApoI
 121 GGACAAACCAACAATAGAATCGAGTGAAGAAAAATGCTTTATTTGTGAATTTGTGATGCT
 CCTGTTTGGTGTGATCTTACGTCACTTTTTTACGAATAAACACTTTTAAACACTACGA
 G Q T T T R M Q * K K C F I C E I C D A

May 27, 2000 17:14 ..

Eco57I HindIII BaeI

80 human 60

TTGCCCTCTGTACTATGGATGAAGCTTCAGAAACATCTTCCTGCCCGTCTGACTAA
ACCGGGGACTCTGATACCTACTCTCGAAGCTCTTGTAGAAGGACGGGCAGACTGATT

from sequence in 1.

D

BamII
SacI

BamHI
BstYI

SV40

CCGAGCTCGAATTAGCTTCGAGGGATCCAGACATGATAAGATACATTGATGAGTTT

TGGCTCGAGCTTAATCGAAGCTTCCTAGGTCCTGACTATTCTATGTAACATCTACAA

BamI

121 SACAACCCACAAGTAGAATGCAGTGAAAAAATGCTTTATTTGTGAAATTTGTGATGCT 18

SV40p2A

CAAAAAAGCATTTGGTAATATTCAGCTTATCTGCTATGTGTGTGTTAAACGTAA
 RleAI DraI
 TTTATGTTTCAGGTTTCAGGGGGAGGTGTGGGAGGTTTTTTAAAGCAAGTAAACCTC
 AAATACAAAGTCCAAGTCCCCCTCCACACCTCCAAAAAAATTTCTGTCATTTTGGAG

pUC19 vector

BsmBI

ACCTGTGACACATGCAGCTCCCGAGACGGTTCACAGCTTGTTGTGAAGCGGATGCCG
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
TGGAGACTGTGTACGTTCGAGGCTCTCGCATGTGTCGACAGACATTGCGCTACGGC

42

GGAGCAGACAAGCCCCTCAGGGCCCTCAGCGGSGTGTGGCGGTGTGGGGCGCAGCCA
421-----48
GTCTGTTTCGAGCAGCTCGAGTGGCCCAACGCCACAGCCCCCGTCTGCT
HaeIII

rI
 I BsaAI 10C19 (tw 499) TatI BseSI BstAPI
 ApaLI NdeI
 CCGAGTCACGATCGGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTG

GGTCAATCATGTCCTGCTCTAATCATGACTCTCAGTGGTATACGGCAC
HaeII EarI
SapI
CTCAGATGCGTAAAGSAGAAAAATACCGCATCAGGCGCTCTCCGCTTCCTC

TGGCGTGTCTACGCATTCTCTTTATGGCGTAGTCCGCGAAGGCGAAGGAG
 TaqII BsiEI BsrBI PciI (rev)
 TACTGACTCGCTGCGCTCGGTCTGCGCTGCGGCGAGCGGTATCAGCTCACTCAA

FTGACTGAGCGACGCGAGCCAGCAAGCCTCCGCGCTGCCCATAGTCGAGTGAGTTT

AFIIII
BspLUIII

GTGTAATATCCGGATATCCAGATATCANGGGATACGCAGGAAAAAGCATGTGAGCAA

CCATTTATGGCATTAGTCTCTTAGTNCCTATTGCGTCTCTTTCTGTACACTCGTT
CCACCAAAAGCNCAGGACCAAAAGGACGCGGGTGTCTGGGGTTTTTCATAGGC
GGTGCTTTTCNGTCTCTGGCATTTTTTTCGCGCCCAACGACCGCAAAAGTATCG

328. 702 GGGCTGNCGAGCATACAAAAATTGACCTCAAAT 818
AGCGGGGACNGCTCGTATGTTTTAACTGGAGTTTAA



Saturday May 27th

tEpoR ~~construct~~ construct

Returned from
West Coast trip with
FitzMurray (#4) and
with ~~Goos~~ ^{Goos} ~~Random~~ (#3)
Super!

The ~~146. seq~~ ^{146. seq} J'139 Red Epo sequence shows
the function between mouse Bactin and tEpoR
perfectly.

The ~~148. seq~~ ^{148. seq} and ~~149. seq~~ ^{149. seq} J'133 Blue EpoR
and Blue 2 sequences show the function of
COOH terminal end of truncated Epo R with
the poly A addition sequence from SV40.

~~The~~ The safest cutting sites for removal of poly A
addition sequences are probably ^{at least 2} Kpn I, ^{3 sites} Sac I and ^{2 sites} Bam HI
despite their being > single sites. Bsm I is probably
safe also. Test by digestions ± Nde I to excise other end.

To ~~be sure~~ ~~of Nde I~~ ~~can~~ ~~sequence~~

From 334 / ~~149. seq~~ ⁱⁿ there is a length of pUC19 (reverse)
in which the Nde I site occurs ~~at~~ it continues as
pUC19 (reverse) to end of sequence. ~~Will check~~

~~5'~~ ^{my} 5' end (146. rev) is completely homologous to
human Bactin (5' ^{my} promoter Y00474 (hubactin. seq)).

Make a primer for sequencing across Epo RI (over page →)

150. seq is 5' of human Bactin - does not
reach the Eco RI site. Only region (BLASTN) is
the last 38bp in Bactin published (GENBANK) data.

Monday May 29th

bactinepor seq (B101)

Assembled the 8.1 kb B101 sequence. The clear choice of excising the [*Bactm. tefOR*(~~stop~~)]

Fragment is partial Kpn I, change to convenient for insertion into a pSKB variant e.g. pSKB1.Am II

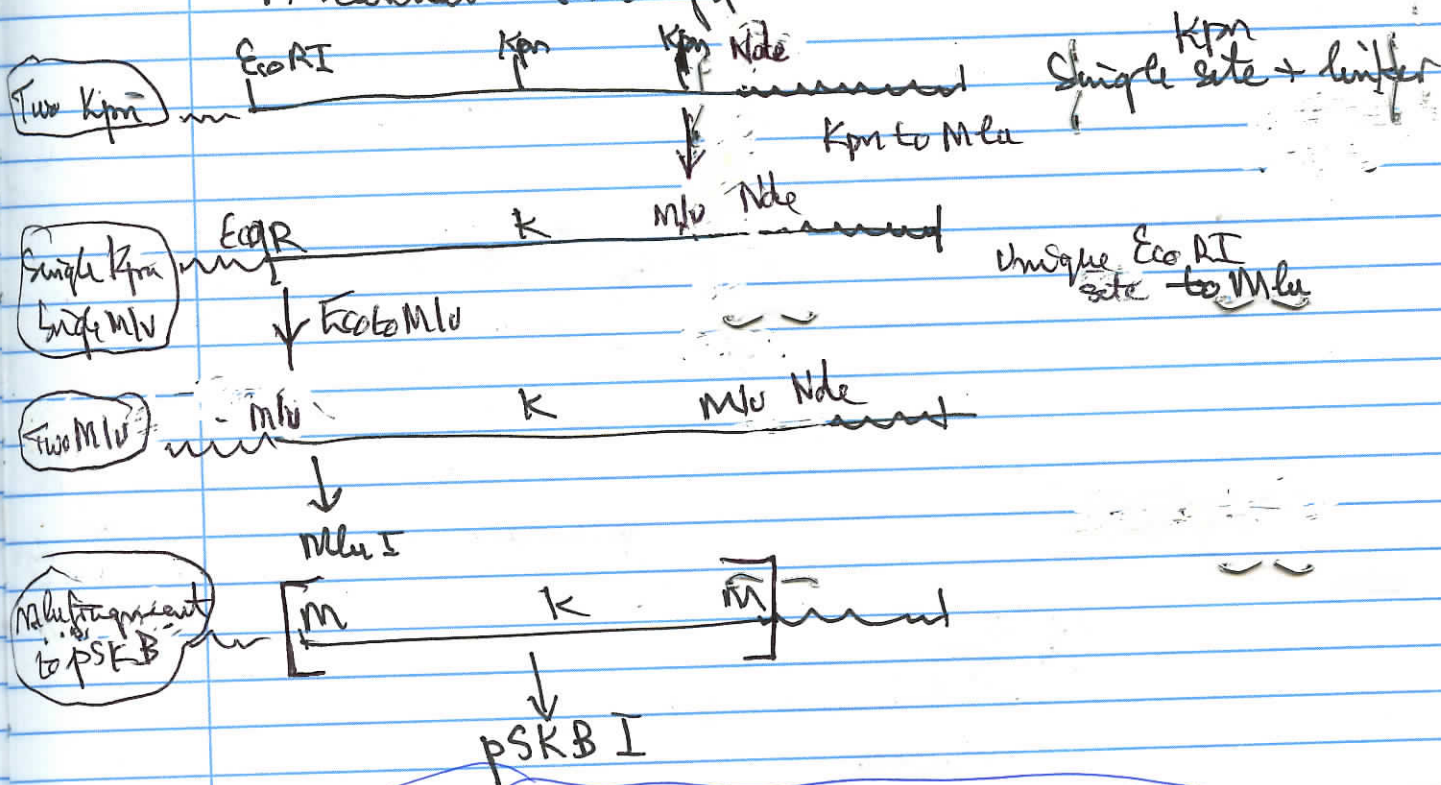
Point #1 Keep P_{me1} for linearization - it is not in the backtrace segment

$D = 40$ (all T) P_{me1} on classic input

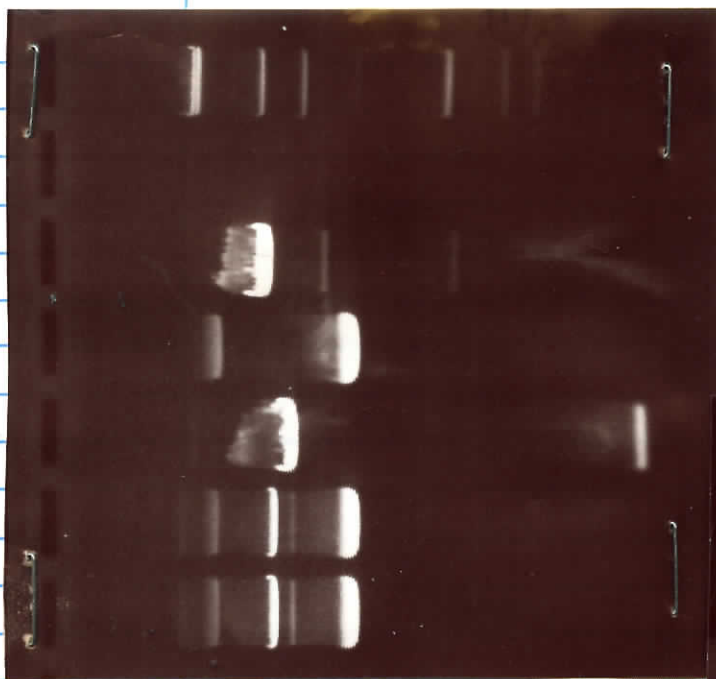
Point #2 { Mlu I & Not I are cloning sites
Apo II

Only survivor

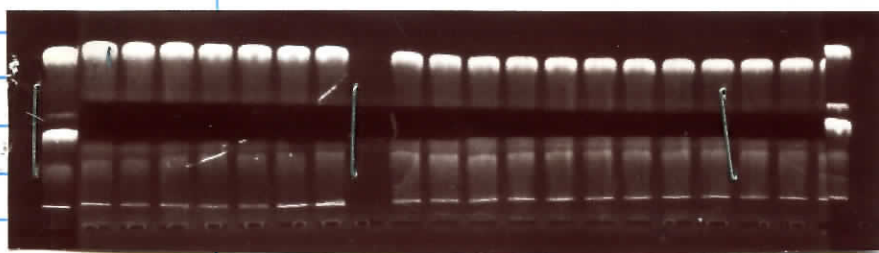
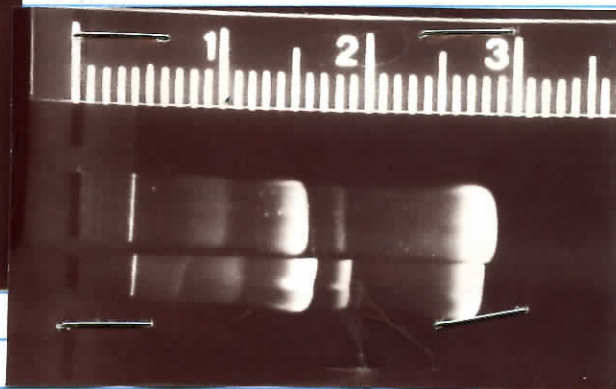
A reasonable strategy is:—



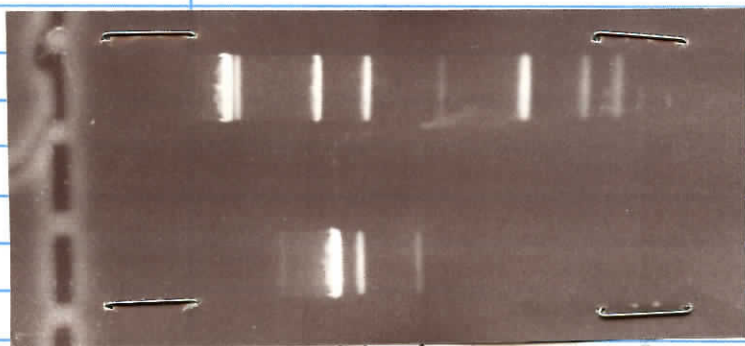
Dont forget the mRNA stabilizer
use PivAI (Age I) as cat



①
Conclusion Sites as expected,
 but kpm a little slow.
 Repeat at 12 min. &
 prepare 1 cut from 6 min & 12 min



② Good cut - but
 will include 2 cut
 material also.



③ Conclusion:
 Yield & purity
 are adequate for
 proceeding.

③ 0.8% Hx4 Max
 0.8% on 11.0 cm
 off 1.2 cm

Dye	10+
w	10-
AG MM	10-
w	10-
5+10 J145 cut	10-
w	10-
Dye	

↑ ↑
 Single cut / Double cut
 Unknown (inverted)

Wed. May 31st

bachinepor Kpon $\rightarrow$ Mlu

Check of sites - expect 2 Kpm, 1 Eco, 0 Mlu

Take 15 μ l (10 μ g) J145 B101
 + 20 μ l 10 $\times$ R1 salts
 + 165 μ l ddw

J 145
B101

BIOL EPO R
SHa50
0.65 μ g/ μ l

37° 9:50am

Prewarm to 37

$$C = 1 \mu F (10V)^{20V}$$

✓ 100%

$$+ \frac{1}{2} \mu(100)$$

✓ + $\mu(100)$ ✓
 blue I
 hot 19 (100/μ)

10 ml

$\frac{1}{2} \mu\text{F}$ 100V $50(20\mu\text{F})$

85 ~~90~~ pul

✓ a 3 min 16 min

75°-80°C

5'145
eco

5'145
m-lu

5 143
Kpm

5'14.5"
Kpn B

5145
Kpn 6

All work prior to running
 - ~~must be off 1:10 pm~~ 0.8% H₂ max

Repeated with
12 mins.

5'145
Kpm 12

~~on 4:12 pm
off 8:30 am
1.5 V / am
no jam~~

②

Diagram illustrating the structure of a **Seaplaque** (likely a **Seaplane** or **Seaplane**).

The diagram shows a vertical structure with a central column labeled **Seaplane**. To the left of the central column, there are two rows of slots, each labeled **4 slots**. The top row is labeled **1st** and the bottom row is labeled **2nd**. The bottom row is also labeled **Kpm**.

The diagram is drawn on lined paper.

~~Handwritten scribbles and symbols, including a large 'X' and various illegible marks.~~

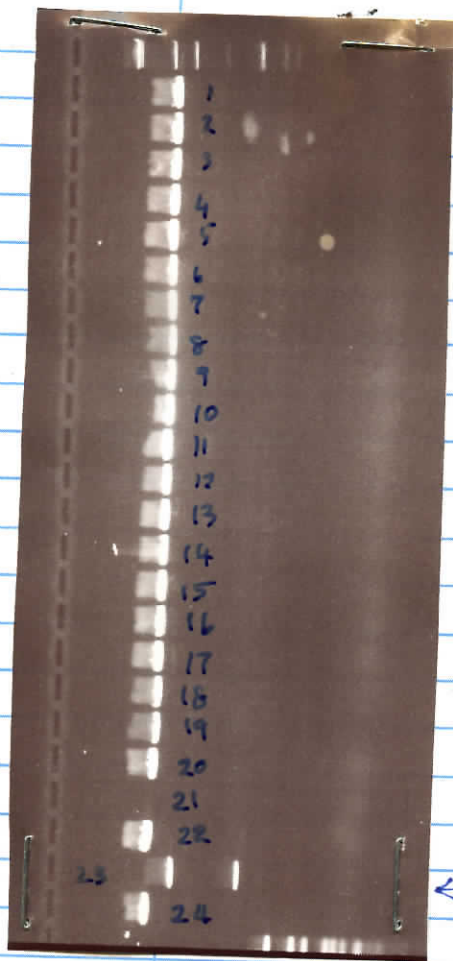
12 slots } $\begin{pmatrix} 1 \\ 4 \\ 12 \end{pmatrix}$

Agarose

Second blue: $\frac{1}{2}$ way
1st blue & yellow: off

Recovered into 2.8ml after 40h/1hr
IAA. Stored ~2ml. Processed
800µl to 50µl 0.001M EDTA

J'145
Single cut
blacktop

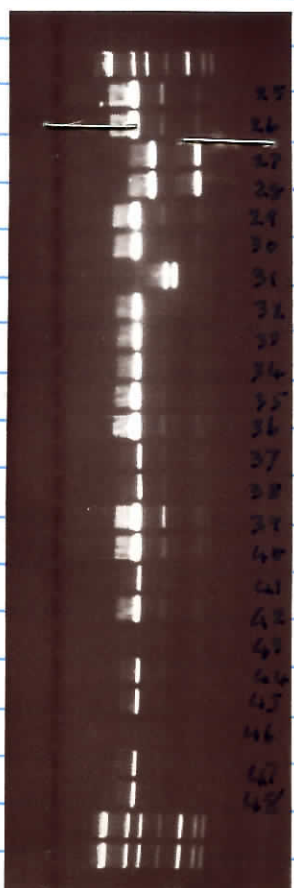
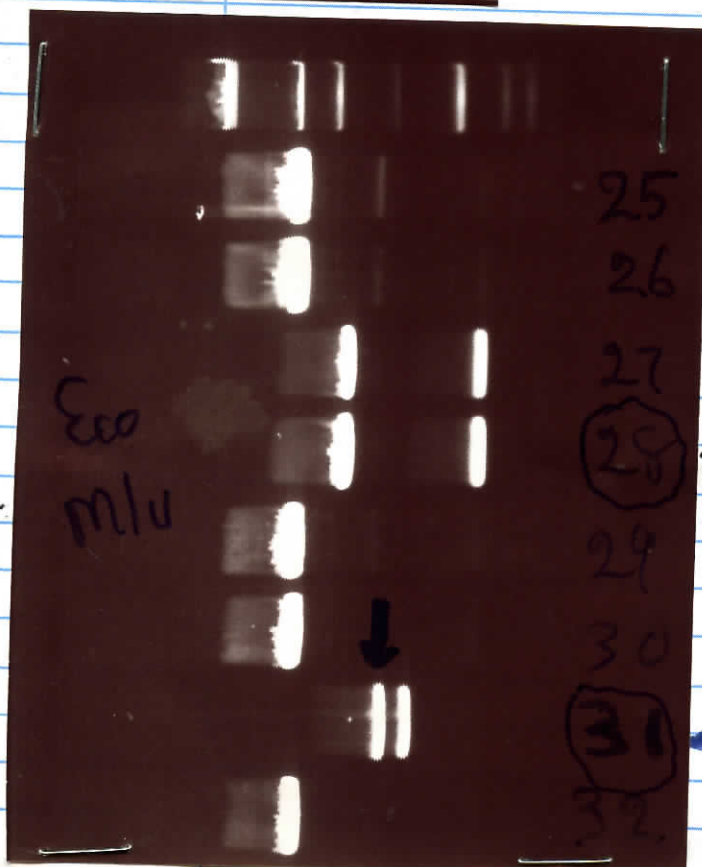


① ? Error may be correct
 Conclusion: Too many starting material - 3!
 Only one can be used - it gives $\sim 6.5 \text{ kb}$ + $\sim 4.5 \text{ kb}$
 This is incorrect site - need $\sim 5 \text{ kb}$ ← + ~ 4.5
 Retransform & try again - ligⁿ may have been poor. Other wise re-purify sample cuts.

Eco Mlu

② ? Error may be correct

Eco Mlu



② Conclusion:
 #31 is correct.
 Proceed.
 ③ ? Error may be incorrect

① 2
 ② 1
 ③ 2
 ④ 1
 ⑤ 1
 ⑥ 1
 ⑦ 1
 ⑧ 1
 ⑨ 1
 ⑩ 1
 ⑪ 1
 ⑫ 1
 ⑬ 1
 ⑭ 1
 ⑮ 1
 ⑯ 1
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Ji
 3
 f

3.1
 kb
 1:30pm
 4:00pm
 0.8% HX4
 RNase 3pc/100
 ~3V/cw

Thurs. June 1st
 backinfor kpa → Mlu (contd.)

See Max
 4pm
 Dye
 25
 48
 Ag
 Ag
 Dye
 contd

Dye
 Water
 100 mM
 ① 5'147
 ② LIG W
 23
 24
 Acry
 W
 Dye

Take 10 µl of 5'145 single (kpa) cut backinfor

- ✓ 1.4 µl of p95 10x ligation buffer
- ✓ 1 µl of 5'142 KPN Mlu linker
- ✓ 1.4 µl of T4 ligase (dilute) lot 970

15° 1:45pm to 3:30pm, R.T. 3:30pm to 4:45pm, 37° 4:45pm to 5:30pm

Kept.

Transformation

To cold

DH5α 5'137 80 µl ✓
 5'147 lig. 5 µl ✓

0° 5:30pm to 6:20pm

42° 90secs

Plated Rest, 10 µl, 5 µl, 20/16, (5/16)

42-43°
 4:37pm to 5:13pm

24 X Mary
 24 NZY 37° 6:30pm

Sun 11:15am

Sat 37° 5:20pm

Fri 37° 6:30pm

5'147 ① - 24 NZY (X Amp) Poor spreading, plated dry & error.
 37° 3pm - 9:30am 5'147 25 - 48 Picked 11:15am 37° - to
 proceeded to 50 µl. Diagnose with Eco RI and Mlu I

Take 50 µl 10x RI salt

X 435 µl 2dw

X 5 µl (100 u) Eco RI lot 28 (20 u/µl)

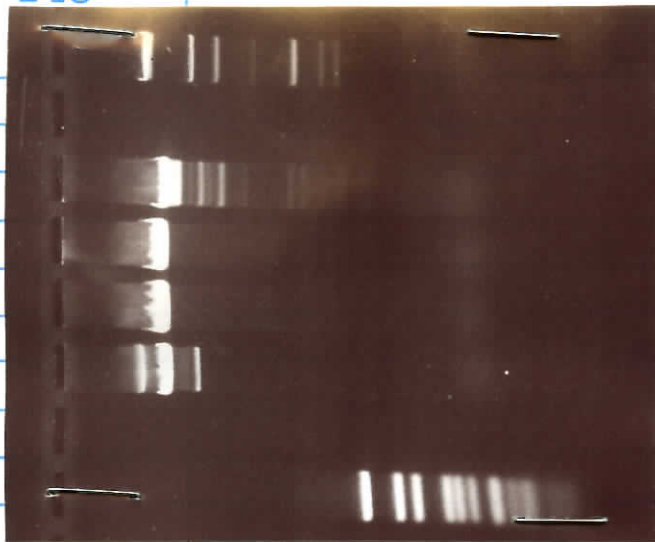
X 10 µl (100 u) Mlu I lot 19 (10 u/µl)

20 µl + 1 µl minus ① - ②

25 - 48
 37° 1:40pm
 2:40pm

37° 12 noon - 1:15pm

3:30pm Picked another 48 49 - 96. growing singly. Proceed in
 pairs of 3 ~ will screen 72 in this way. 37° 4:15pm - 9am Mon



①

Conclusion: All are as expected.
 Glad we don't need to use Age 1!
 Pme I linearization will
 be slow - but may be better in k⁺

ONE
 1
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 100

Fri. June 2nd

Choice of pSKB1.AVR II insertion site
for mRNA destabilizing sequence

Map of pSKB1.AVR II assembled June 1, 2000
shows Mlu I cloning site, as expected
and the Pvu AI (Age I) site at end of 3' homology.
Two cut map also shows 2 adjacent Sal I sites
surrounding the Pvu AI. Since Sal I is more versatile
I will use these for insert of destabilizer, linearization
will be Pme I.

Destabilizer
[Sal I Destabilizer Avr II] Sal I...
or ?
For orientation

Bye (1)

Agarose

W

Age I

Sal I

Mlu I

Pme I

W

Agarose

W

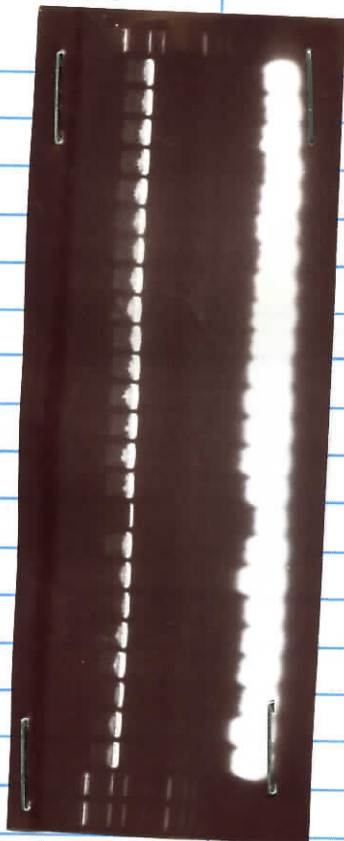
Bye

Since the oligos will be large, left sites in pSKB1.AVR II

Take 6 μ l (2 μ g) J149 pSKB1.AVR II (Seigo's J69 is a min prep)
✓ 70.5 μ l ddw
✓ 8.5 μ l 10x R1 calls

20 μ l $\downarrow$ 20 μ l $\downarrow$ 20 μ l $\downarrow$ ~20 μ l $\downarrow$
2 μ l $\downarrow$ 1/2 μ l $\downarrow$ 1/2 μ l $\downarrow$ 2 μ l $\downarrow$
[Age I] [Sal I] [Mlu I] [Pme I]
lot 7 (20 μ l) lot 31 (100 μ l) lot 19 (100 μ l) lot 13 (40 μ l)

37° 3:30 pm to 5 pm



(1) Conclusion (#31) - I'd have checked R1 digest
2 perhaps purified the fragment,
#28 may be better - 24 hr. ligation.

Error in linker

Bactinept

~~EcoRI~~ EcoRI $\rightarrow$ MluIEco

mini #31

Byg
w
AGMM

12

#31



21

7

A

Br

like 3rd of J 147 Mini #31

✓ 2nd pl of 10x R1 salt

✓ 15 ml of H₂O

✓ $\frac{1}{3} \mu R (100)$

Eco RI lot 28

306:06 to 8:05 pm

Heat in act d $80^{\circ} - 83^{\circ}$ 10 min

MluI

diges

+ 2.6 µl p95, 10x H_2O buffer

 $+ \frac{1}{2} \mu R^2$

+ 2 μ l T4 ligase (dilute) lot 970

15^c 9:15 am (Tues) RT 12:34 32° 1:27 pm - 2:10 pm

When making prep gel, cut with MluI and
something in the flanking to overlap.

Transformation

DH5 α (C'231)

Q. 2

151 LIG (3)

~~5 pe~~

2:15 pm to 3:17 pm

90 sec	42°
--------	-----

Plates 1x Amp NZY

~~18, 20, 5, 26~~ / 16, 8 / 16

37° 3:30 pm

12 30pm

Shaking at
2-18-20

Picked J'151 (31) ① - ② 1xN2YAmf

37 3:40 PM

7. Hold, pending sequence analysis of 150 seq. May be mini 2B is better bet.

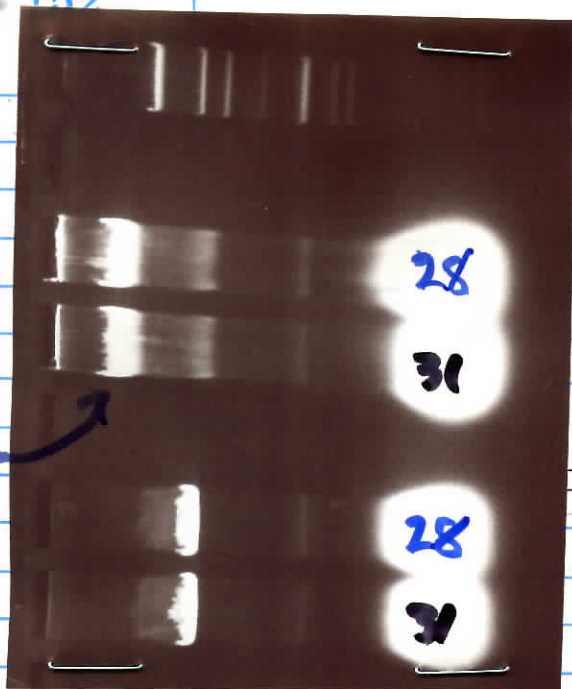
Take 50 μ l 10 $\times$ EcoRI salt.

370 12.37 ^{pm} to 1:54 ^{pm}

✓ LiAlH_4 and LiAlH_4

$$\sqrt{10 \mu\text{e} (1000) \text{ M/V I hot } 18 (100 \mu\text{e})}$$

20 μ l + 1 μ l each #31 (5) min

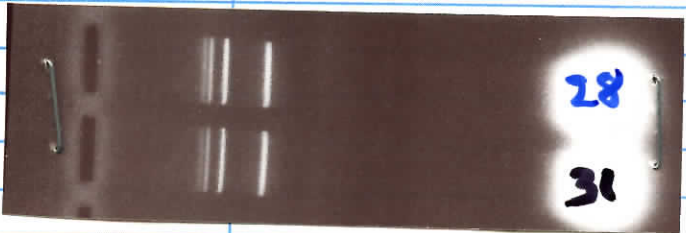


① Canduria : lig^{ns} were fine. Cut is fine. Make fragments if #28 fails - since there is appreciable ssDNA. However, the RI ligated to itself! Error in the linker!! Corrected

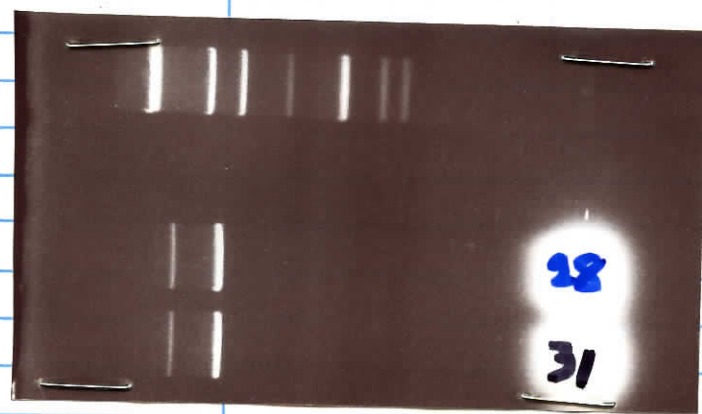
2593/ Oligo Name: J'152 ECOMLUECO
 5' AAT TCA CCG GTTG 3'

} J'153 EcoRI digests

Self ligated!



} See J'161 for gel
 ligation with correct linker is fine
 tho' still some multimers
 Perhaps need to ↑ conc. of linker in future



} See J'162 for gel
 Big improvement with ↑ in linker

Tues. June 6th

Mini #28 bacteriophage Eco RI $\rightarrow$ Mlu I Eco RI

Take $\checkmark$ 3 μ l J'147 Mini #28

$\checkmark$ 2 μ l of 10x RI salts

$\checkmark$ 15 μ l of L₁W

$\checkmark$ $\frac{1}{2}$ μ l (10v) Eco RI lot 28 (20v/ μ l)

J'153
(28) Eco RI

37° 1:55 pm to 3:35 pm

Heat inactivated 10 min 80° $\checkmark$

$\checkmark$ + 2.6 μ l p95 10x L₁W buffer

$\checkmark$ + 1 μ l J'142 ECOMLU ECO linker

$\checkmark$ + 2 μ l T4 ligase (dilute) lot 970

To cold 3:50 pm Tues

J'153
LIG (28)

12:30 pm Wed - to cold

Saved enough
of each ligation
for a new transform
at 2 to 3 μ l

Transformation

DH5 α C'23 $\checkmark$ 80 μ l

J'153 (28) L₁W $\checkmark$ 5 μ l

0° 12:40 pm to 1:15 pm

42° 90 sec

1xNZY Amp $\checkmark$ 20
R, 20, 5, 20, 5
16, 16

37° 2 pm Wed

9:30 am Thurs

Picked J'153 Mini ① - (24) to 1xNZY Amp RT 10 min ~ 4 pm
37° 4:15 pm

Check on ligation:

Take $\checkmark$ 8 μ l 10x Eco RI salts

$\checkmark$ 70 μ l L₁W

$\checkmark$ 1 μ l (40 v) Eco RI lot 28 (20v/ μ l)

Blue
J'153
Eco (28)

40 μ l

2 μ l J'147 (28)

40 μ l

2 μ l J'147 (31)

Black
J'153
Eco (31)

37° 4:30 pm to 5:30 pm - Frozen

Inactivated 10 min at 80° C. $\checkmark$

Tues. June 6th

More backtime for sequence

Oligo #	5'	Oligo Name	J'155 Red Actin	3'
Date made:	CAIC	CIT	GCIC	TTIG
LENGTH: 19	CITIG	A		
% G: 56%				

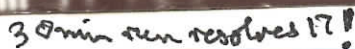
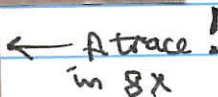
Continued same way as Red Actin J'142
(in the direction relative to 150.seq)

AarI → 150.seq →

```

601  AACTCAGCCCAAGGGGACTCCACCTGCCTGGCTGGGACTTTTGTCTTTTCCCTNCAACCT
      +-----+-----+-----+-----+-----+-----+
      TTGAGTCGGTTCCCTGAGGTGGACGGACCGACCTGAAAACAAAAAGGGGANGTTGGA
      660
661  TTGCTTTGGTGACGCGCCGTTGCAAGGNCNCAGCAACCAACTTTACAAGGCAAAAC
      +-----+-----+-----+-----+-----+-----+
      AACGAAACCACTGTGGGGGTGGAACGTTCCNGANGTCGTTGGTTGAAATGTTCCGTTTGG
      720
      PFLMI
      44+16 Red Actin
      J'155
721  ANGGACCACTCCTTGGCCTCCGAAAACAACCAACCCAGGACCTTGGCTTGGCACTGANG
      +-----+-----+-----+-----+-----+-----+
      TNCCTGGTGAGGAACCGGAGGCTTTTGTGTTGGTGGTGGTGGGAACGGAACCGTGACTTNC
      780
  
```

151. Seq set up by Sylvie with B101 & J'155 Red Actin
- no useful sequence.



Precipitate, cut with
Sal & H₂O & purify

(2) ---
Can see the
Xho/Sal cut !!
2 $\frac{1}{2}$ scaps is v. good
at this size
good cut.

(9) (3)

ON 2:12 PM
ON 2:42 PM

J156 Sal/Xho

J157-PCR

Dye

W AgMM

J161 CIAA SALCIAF Dye

J156 ARE S/X ACR MM

W

ACR MM

W

Dye

(d) Dispensed MIX (less tag) to 5x45 µl + 1x25 µl. Into machine at $>90^\circ$.
Pump off. Allow to cool to 85°C , then ~~added~~ added

4 μ l Tag to each 95 μ l tube & returned program 60° etc. Removed 25 μ l = 5 min

J'167
AREPCR

Take 380 μ l of 157 ARE PCR + 38 μ l 8M NH_4Ac + 1.05 ml EtOH Use this for 2nd and 3rd

Freeze. Cent. Dry & back into 100 μ l 0.001M EDTA at J 15 + PCR conc. $\frac{1}{2}$ μ l (10) Sal I + $\frac{1}{2}$ μ l (10) Xho I
Take 80 μ l J 157 PCR conc + 9 μ l 10 KRI salts + 1 μ l (10) Sal I + $\frac{1}{2}$ μ l (10) Xho I
J 15 Sal/Xho 32° 6pm to 10:45pm $\frac{1}{2}$ μ l 10 u / μ l $\frac{1}{2}$ μ l 200 u / μ l
Tues 31 Jan 3B

⑤ Prop. gel Cut & recovered into 200pl as J156 ARE S/X ③ Control it! Trick recovery 100pl.

Tues. June 6th (ERROR) → 157

~~y~~

sats 1.5%

+1

M

"25" J157 No Top (A)

J157 PCR are seq.

To insert ends. To

W ACAMM

Add shorter if unsuccessful

Message Instabilität

are seq. is 69bp from c-fos that are mRNA destabilizing
To insert into pSKB1. AVR will require Sal or Xho
ends. Following two long primers will be used first:

$\frac{96}{100} \times 100 = 96\%$

136
al/Xho
157 PCR
ACRMM
W
20-8 O.D. units
into 500 μ l
(w 2 μ g/ml)

June 6, 2000 15:57 ..

Col I

GGTCGAC

J157 Blue Arc Hin4I

ttttattgtgtttttaattattttataagatggattctcagatatattatatttttttt

aaaataacacaaaaattaataaatattcttacctaaagctctataaatataaaaaataaa

tattttttt

61 ----- 69

ataaaaaaa

3416

3425

J157 Blue Arc

X40 I

Thus missed
19 bp
from
3' end

Oligo # 25895 5' Oligo Name: J157 Blue Arc 3'

Date made:

G	G	T	C	G	A	C	T	T	T	A	T	T
---	---	---	---	---	---	---	---	---	---	---	---	---

LENGTH: 57

G	T	G	T	T	T	T	A	A	T	T	A	T
T	T	A	T	T	A	A	G	A	T	G	G	A
C	T	C	G	A	G	A	T	A	T	T	A	T

% G-C:

Oligo # 5' Oligo Name: 3'

Date made:

--	--	--	--	--	--	--	--	--	--	--	--	--

LENGTH:

% G-C:

Oligo # 25896 5' Oligo Name: J157 Red Arc 3'

Date made:

C	C	C	C	T	C	G	A	G	A	A	A	T
---	---	---	---	---	---	---	---	---	---	---	---	---

LENGTH: 44

A	A	T	A	T	A	A	T	A	T	C	T	G
G	A	A	T	C	C	A	T	C	T	T	A	A

% G-C:

①b) As safety, make the following two secondaries.
The others won't amplify - only anneal!! & fill in.

Component	Volume
25µl	
Template	
20X PCR	1.25
dNTPs	0.1
DMSO	2.5
Blue	0.5
Red	0.5
Tag	1.0
Water	2.5

25932

Cligo Name: J157 Blue 2

Cligo =

Date made:

LENGTH: 19

% C-C: 42%

25933

Cligo Name: J157 Red 2

Cligo =

Date made:

LENGTH: 21

% C-C: 29%

2x250
litres
2x10Tao

PCR if
(10) Xho I
200 μl
38

Water 25

(C) Take $n = 20$

J157 Blue Amp template 1 μl ✓
J157 Red Amp template 1 μl ✓
J157 Blue 2 (primers) 10 μl ✓
J157 Red 2 (primers) 10 μl ✓

3157 Heat to 90° & slow cool. Then add
 ✓ 20xPCR 25µl 20µl lab Tag at >70°
 ✓ dH₂O 400µl photo 5 tubes with 20µl frozen
 ✓ dNTPs 2µl
 3157 DMSO ✓ 50µl ~~Not to be used~~
 Then 90° 1min 40° 7sec. 60° 5min x 35 cycles



① Conclusions As expected, only the 2X colonies have plasmid. But didn't expect no site change.

The total transformation was very poor. Run prep. gel on ligations or run more samples.

Repeat transformation with J159 cells ← New lot, proc 6/17
+ rest of 28 & 31 J159 lig. 2
0° 4:13 pm to 4:53 pm

✓ ≤ 42° (not recommended) 90 sec
(Wet plates) Plated 2X Amp R, 20, 5, 20, 5, 16, 5, 16

(28)

(31)

(5/16)

R, 20, 5, 20, 5, 16

(5/16)

37° 5:20 pm

>50 !

Save, but re-transform with J168 ligations

Fri. June 9th

Repeats of Eco → Eco Mlu Eco on (28) & (31)

Dye
W
AGMM

(1)

(28) Mlu

(10)

(28) Cold room 6:37pm

(13)

(28)

(28)

(31)

(28)

(31)

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(31)

Heat inactivated EcoRI digest of (28) & (31)

J'153

J'153

J'153

10x p95

J'152 ECOMLU ECO linker

T4 ligase (dilute) lot 960

(28)

(31)

Transformation

(28)

(31)

(28)

(31)

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(28)

(31)

DH 5α C'23

J'159 lig?

AG

AG

Dye

3:08pm to 3:39pm

42° 90sec.

plate 20, 5, 20/16

NZY

37° 3:55pm to 10:30am

Not so but NM had trouble with that also

5/31/00 probably

1x amp

5/16 probably

5/20/00

5/20/00

5/20/00

5/20/00

5/20/00

5/20/00

5/20/00

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5/20/00

5/20/00

5/20/00

5/20/00

5/20/00

5/20/00

V. odd result - 1x Amp plates must be too old -

~15 small colonies on (31) 5/16, none on 20/16, 2 big, 1 small on 5, 4 on 20

~5 normal of (28) 5/16 plus wide background of tiny, none on 20/16, none on 5, 9 on 20

Pick the 2x Amp colonies & fill in with 1x largest. 2/8/00 2x Amp plate

(28) (1) - (8) (9) (10) - (12) (31) (13) - (19) (20) - (24)

Picked 11-11:30am

37° ~ 4pm to 8:30

Didn't grow on 2x plates! but grew in 1x liquid culture !!

Data on ligations on J'160, 161 and 152.

Take 150 µl 10x EcoRI salts

✓ 440 µl ddw

✓ 10 µl (1000) Mlu I lot 18 (100 µl)

20 µl + 1 µl of mixed J'159

37° 12:00pm to 2pm

160



① Conclusion. See J'152 for lig. gel
J'161 cuts are fine. Conc. is good.

①

5+

5+

gel
good.

On 4:06 pm
Off 4:56 pm
4:56 4:42 4:12 x

Sunday June 11th
Acceptor for ARE

Salt Salt (CIAP)
PSKB.AVR II

(161)

1) Dye
W
5+10 (28) hig. J159 ✓ 8 µl 10x EcoRI salts
5+10 (3) hig. J159 ✓ 68 µl ddw
✓ 4 µl Seigo's J89 2+3 mini #11 J'99 Red
W 16 µl 64 µl
Pre J'161
Post Sal J'161
W
AgMM
W
Dye
J'161 PRE
J'161 Sal
+ 2 µl (20 u) Salt Lot 31 (10 u/µl) Jan
37° 12:25 pm to 3:35 pm
✓ + 12.3 (1/4 vol.) 5x CIAP buffer
✓ + 2 µl Lab CIAP (8 u)
50° 3:42 pm to 4:17 pm
16 µl ✓
J'161 Post Sal
260 µl 1x POH/HOQ 2x CIAA
+ 6 µl 8M NH₄Ac
+ 165 µl EtOH
J'161 Sal CIAA CIAA
Freeze. Tent. Dry. Back up into 0.001M EDTA 60 µl ds

Wed. June 14th

ARE into PSKB

Take 2.5 µl J'161 Sal CIAA CIAA (of PSKB.AVR)
✓ 12.5 µl (whole) J'156 ARE S/X (recovers)
✓ 2 µl p95 10x
✓ 2 µl T4 Pol (detute) lot 970
Cold room 6 pm to
J'161 ARE hig
J'161 WIG N
PSKB.AVR II
Not much hope. Start again tomorrow.

For
Cancelled

162

9:00am
0.8% Hxt Max

Mon. June 12th

Repeat EcoRI → EcoMluEco (28) & (31)

Photograph
on J152

J162
(28)
ligⁿ

J162
(31)
ligⁿ

5+10 28 ligⁿ
5+10 31 ligⁿ
w
Dye

Heat inact.
J153
EcoRI
digest

18 pl ✓
p95 3 pl ✓
10x 3 pl ✓

18 pl ✓
3 pl ✓

First mix.
Heat to 60°
then

EcoMluEco
linker

J152 5 pl ✓
5 pl ✓

(23 x previous)

room 4:50pm 3 pl ✓

3 pl T4 ligase (dilute) L970

run a gel on this before proceeding.

show a great improvement in ratio of monomer.

	J162 (28)	J162 (31)
159	35 pl ✓	35 pl ✓
162	2 (28) 2 pl ✓	31 2 pl ✓
0.2 30 min	to 3.05 pm	
42°	90 sec	

Plated 2 x Amp NZY 5/31/00
20, 5, 20/16 5/16 (28) 37° 3:20 pm
20, 5, 20/16 5/16 (31) to 8:45 am

1 — 12 to 37° J162
13 — 24 1 x Amp NZY at 4 pm Wed.

ENAD 5m KOAc was not acidified. ~9 Thun
Added 58 pl (from recipe) to each & proceeded
→ 370 pl iso prop. look OK.
50 pl 10K Ribolets, 440 pl dH₂O, 10 pl (100%) MluI L970 (100 pl)
37° with 1 pl J162 mini at 11:33 am to 2:30 pm

(28) #34849 are correct. Use 9 ← (28) [7]

(31) #13 & 16 are correct. Use 13. ←

1 — 12 to 37° J162
13 — 24 1 x Amp NZY at 4 pm Wed.
ENAD 5m KOAc was not acidified. ~9 Thun
Added 58 pl (from recipe) to each & proceeded
→ 370 pl iso prop. look OK.
50 pl 10K Ribolets, 440 pl dH₂O, 10 pl (100%) MluI L970 (100 pl)
37° with 1 pl J162 mini at 11:33 am to 2:30 pm
(28) #34849 are correct. Use 9 ← (28) [7]
(31) #13 & 16 are correct. Use 13. ←

Mar - June 19th

Renamed J'162 (9) as J'162 MluEpo

derived from J'153 Eco (28)

Lo 970
3:20pm
to 4:45 am
>30

MM
MLUI-
162
mi)

Boorum & PeaseTM

Columnar Books • 10 3/8 in. x 8 3/8 in.

150 Pages	300 Pages	Ruling	Width of Desc. Space	Units Per Column
Record Ruled				
21-150-R	21-300-R	Record	-	-
Columnar / Ledger / Quad Ruled				
Single Page Forms				
21-150-2	21-300-2	2 Columns	3 3/8"	10,000,000
21-150-3	21-300-3	3 Columns	3 3/8"	1,000,000
21-150-4	21-300-4	4 Columns	2 3/8"	1,000,000
21-150-5	21-300-5	5 Columns	1 5/8"	100,000
21-150-6	21-300-6	6 Columns	1 3/8"	10,000
21-150-7	-	7 Columns	1 3/8"	10,000
21-150-DEL	-	D.E.Ledger	1 3/8"	10,000,000
21-150-Q	21-300-Q	Quad-5 Sq.	-	-
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21-150-8	21-300-8	8 Columns	3 5/8"	10,000,000
21-150-10	21-300-10	10 Columns	4 1/4"	1,000,000
21-150-12	21-300-12	12 Columns	2 3/8"	1,000,000



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