Jan. 16th 1994.

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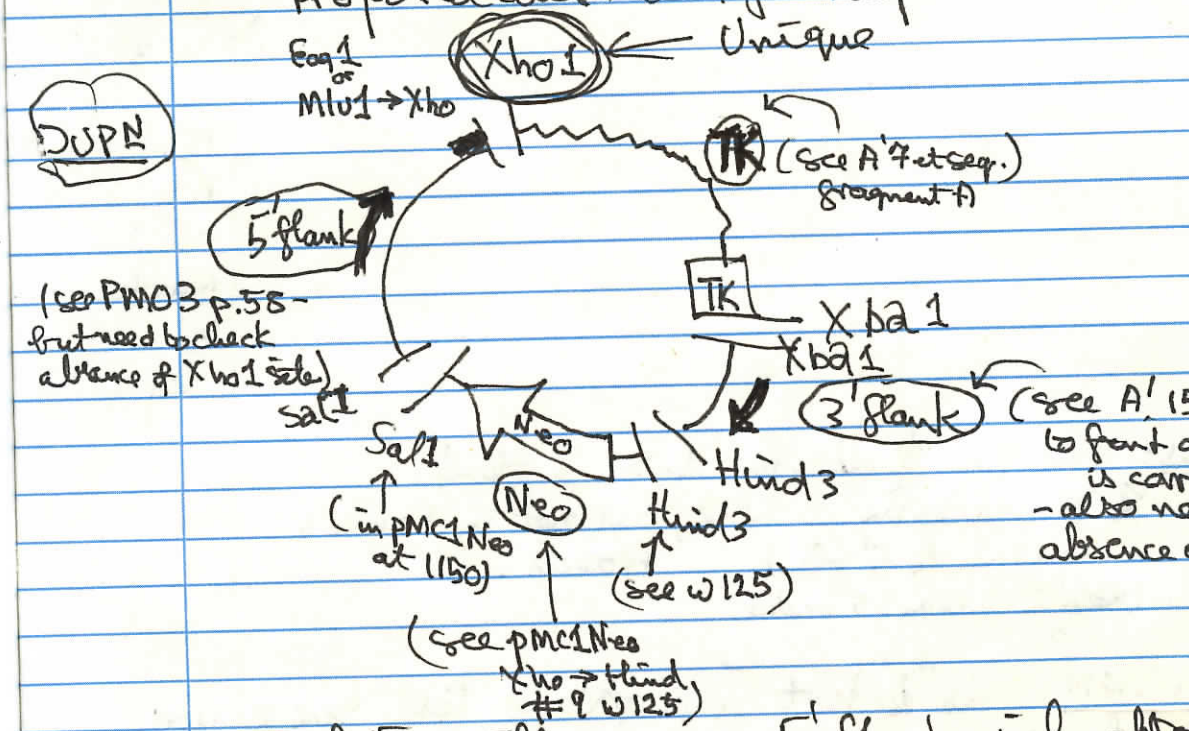
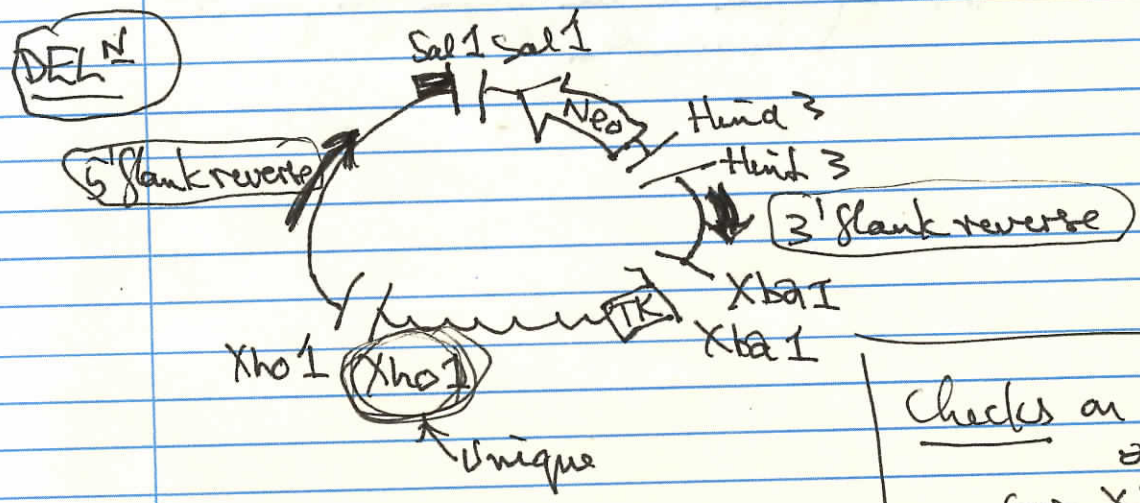
Sun. Jan 16th

ANPRA: DUP (cont'd.)

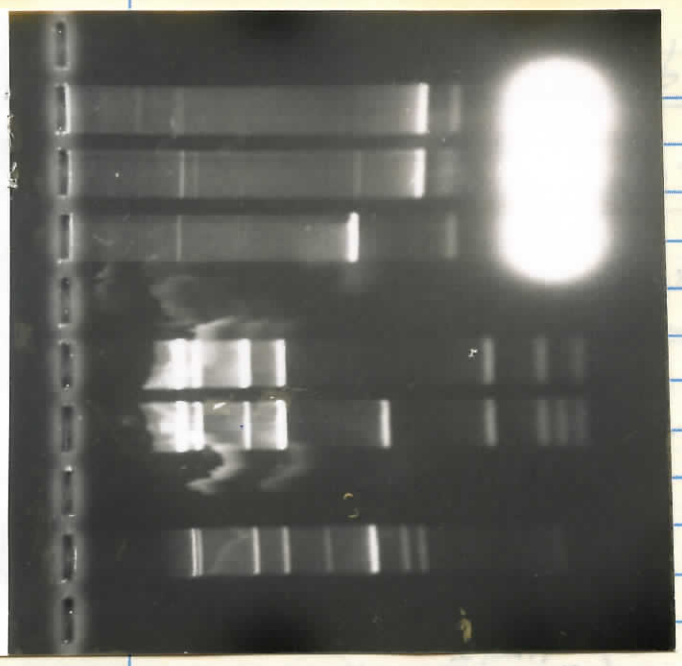
Proposed constructs for dup^N. & del^N.



The deletion will require 5' flank with opposite sites; the 3' flank opposite is already made.



Checks on 5' flank & 3' flank for Xho on next page →



Dye	
Water	
B'3	3'C
B'3	3'X
B'3	3'H
W	
B'3	5'C
B'3	5'X
W	
Marker	
W	
Dye	

Conclusions: No Xho 1 site in 1.3 3' flank.
2 Xho sites in ϕ 2B2, but not in Paula's
cantidate 5' flank. Proceed.
No XhoI sites in Charon 35 vector

CAUTION This may not be a rigorous test since we have to assume the 9.5 kb give 6.5 kb + 2.8 + ~0.6
But the 6.5 could have given 2 × 3.8 + 0.6 – unlikely
Anyway test again when fragment available.

0.8% HX4 MAX
On 3:36pm
Off 3:36pm

Sun. Jan 16 d

Checks on 5' & 3' flanks for Xho sites

3' flank

Take ✓ 6 µl 10XR1 salts
✓ 50 µl ddw
✓ 4.5 µl A'14.8 #26 mini 3' flank

5' flank

✓ 20 µl → ✓ + ½ µl (10u) Hind 3 Lot 45 → B'3 3'H (Digestion control)

✓ 20 µl → ✓ + ½ µl (10u) Xho 1 Lot 26 → B'3 3'X (Test)

Rest → B'3 3'C (No enz.)

37° 12:25 pm → 1:25 pm

Take ✓ 4 µl 10XR1 salts
✓ 26 µl ddw
✓ 10 µl Paula's Charon 35 clone 2B2 (SHS 66, 2 B2, 0.1µg/µl) B'3
✓ 2 [redacted] µl Eag 1 (20u) Lot [redacted]21
✓ 1 [redacted] µl Sal 1 (20u) Lot 47

✓ 20 µl → ✓ + ½ µl Xho 1 (10 u) Lot 26 → B'3 5'X

Rest → B'3 5'C

07183

made: 1-16-94

GTH: 12

C: 83%

5' Oligo Name: OSEAGXHO

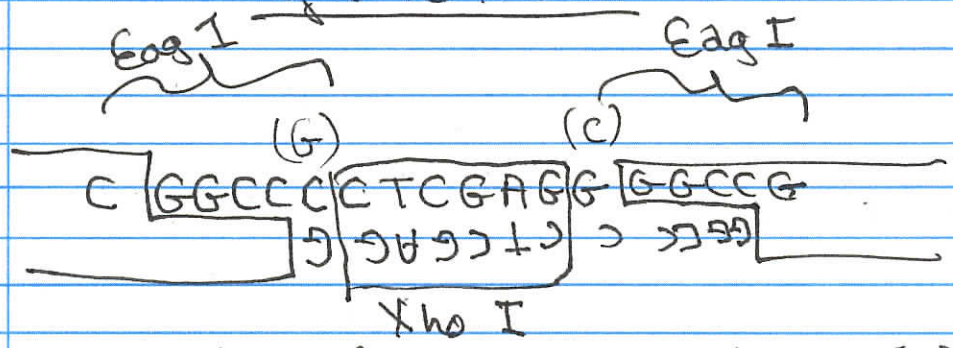
G	G	C	C	C	C	T	C	G	A	G	G	

G 500 Å 0.2 uM NAG-020-500 Prime Synthesis 0 0 4 9 6 0

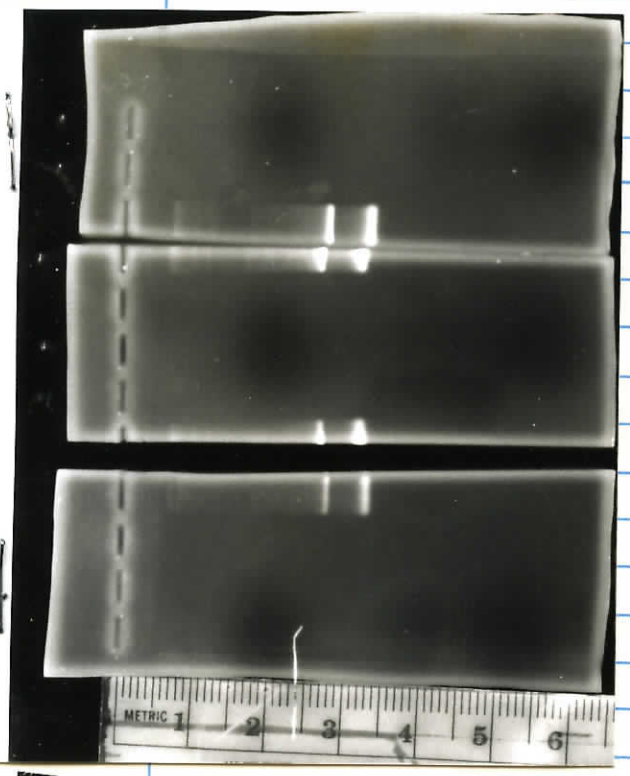
Back up into \mlTE.\XCIAA
Stored as B'5 OSEAGXHO

Sam. Jan 16th

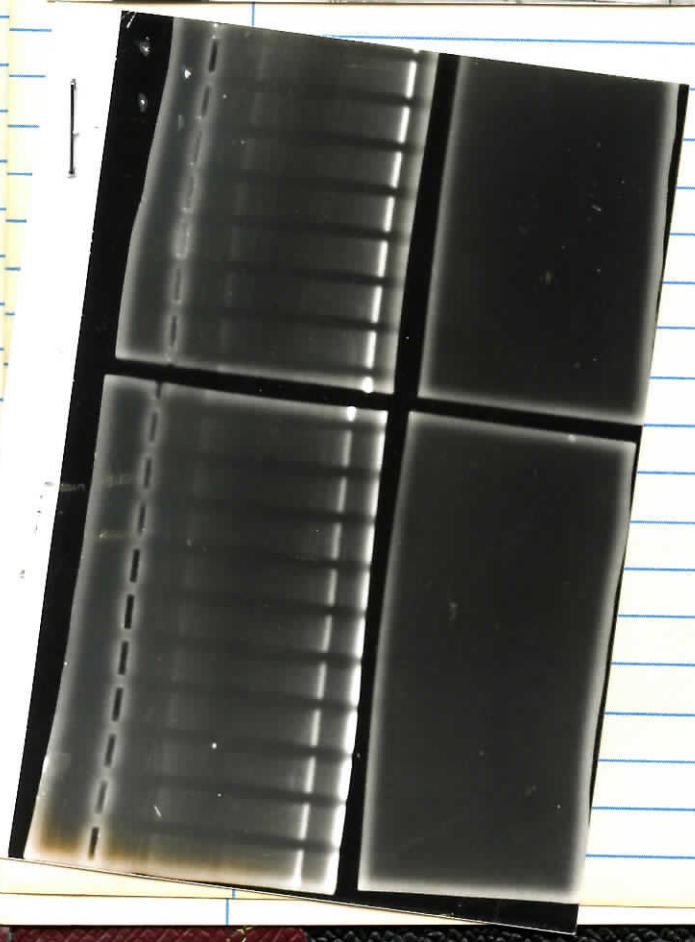
Eag I to Xho I



8 bp overlap. Eag removed. Xho I inserted.



Conclusion: looked like neither cut at all! Prepared fast band (roughly) anyway. Good cut for 148 Blue
– moderate for 155 Red (Black)



Mon. Jan 17th

3' flank preparations

Sylvie made the two orientations in supernatant as

A'148 H1.3X and A'155 29 X/H
o.s. → (#26) o.s. → (#29)

The vector is ori-vector, ~2kb, so can seperate ok.

Take ✓ 21 µl (3 µg) A'148
✓ 3 µl 10x R1 salts
✓ 4 µl ddw
✓ 1 µl (20u) Hind 3 Lot 45
✓ 1 µl (20u) Xba 1 Lot 29

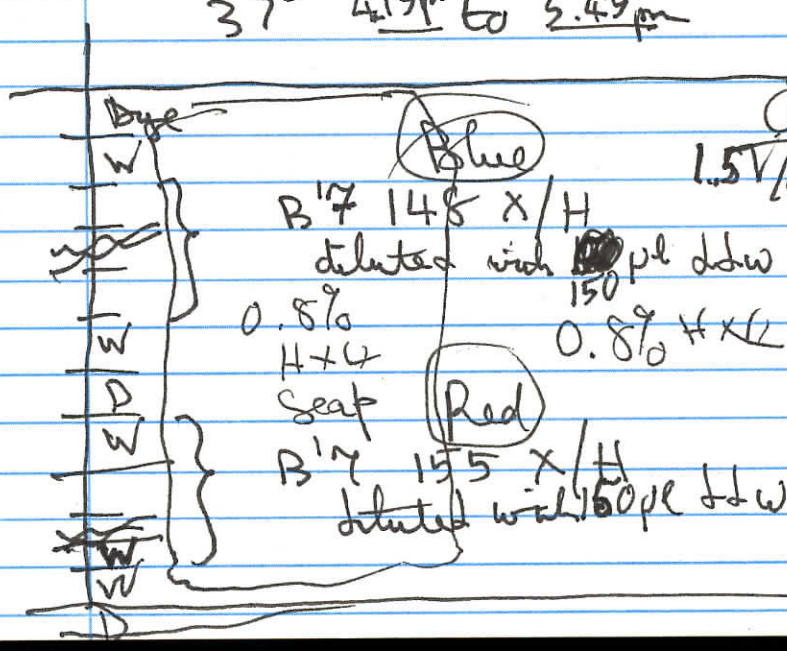
Blue: B'7 148 X/H, 0.1 µg/µl

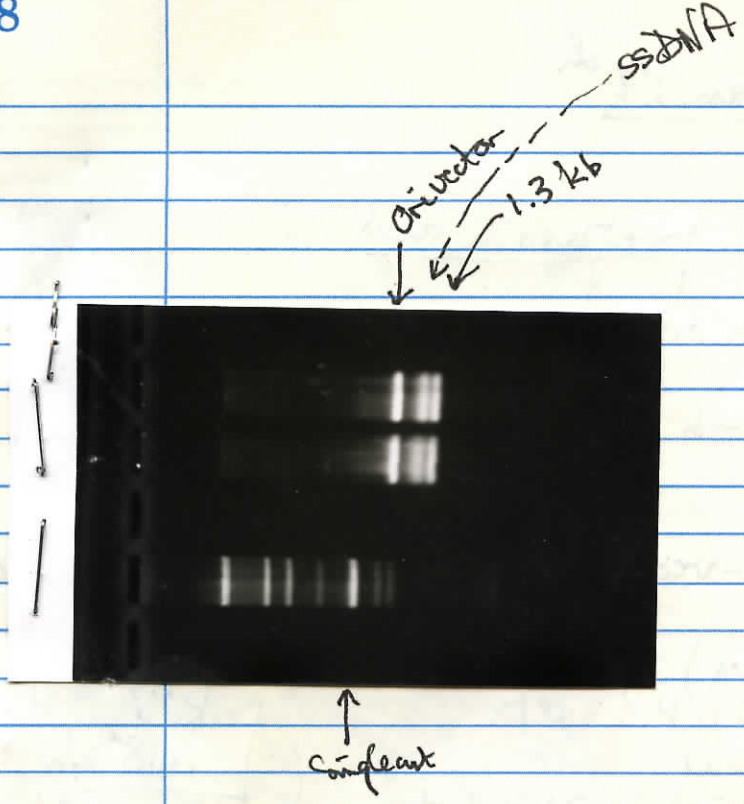
→ ✓ 14 µl (3 µg) A'155
✓ 3 µl 10x R1 salts
✓ 11 µl ddw
✓ 1 µl (20u) Hind 3 Lot 45
✓ 1 µl (20u) Xba 1 Lot 29

Red: B'7 155 X/H, 0.1 µg/µl

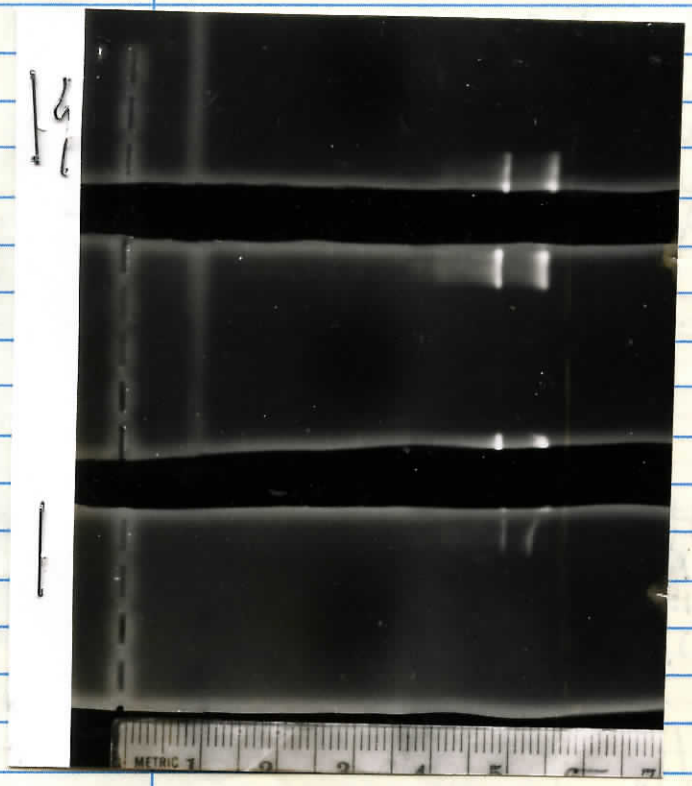
37° 4:15pm to 5:45pm

8.05pm ↑ to 520mA
On 6:35pm Off 10:15pm
1.5V/cm ~1 Max 260mA
(~0.25 µg/sample lane)





Conclusion: Dilution works – or gel is better??
Reaction went to completion, probably with rather a lot of ssDNA.
There is not singlecut plasmid – it migrates at 3.3kb



Conclusion:
Must have been gel resolution all along. Repeat digest & try using 1.2% seap. (1X) on test sample first.
B'q² 37°
10:30 pm → 9.15a

Tues. Jan. 18th

X 11.3 H digestion check

40µl Excess of B'7 148 X/H diluted +4µl 10X EcoR1 salts

37° 8.55am to 10:30am

20µl

B'7 148 X/H dil'd

20µl
+½ µl Hind 3 lot 45
+½ µl Xba 1 lot 29

B'7 148 X/H 2 dil'd

Dye
W
X/H dil'd
X/H 2 dil'd
W
Marker
W
Dye
On 10:47am Off 11:30am
0.8% H x 4 max

Blue

B'9 2

Take 21µl (3µg) A'148
X 18µl 10X R1 salts
X 139µl ddw
X 1 µl (20u) Hind 3 lot 45
X 1 µl (20u) Xba 1 lot 29

A'9 148 X/H

Red

X 14µl (3µg) A'155
X 18µl
X 146µl ddw
X 1µl Hind
X 1µl Xba

A'9 155 X/H

37° 12:30pm to 3.45pm

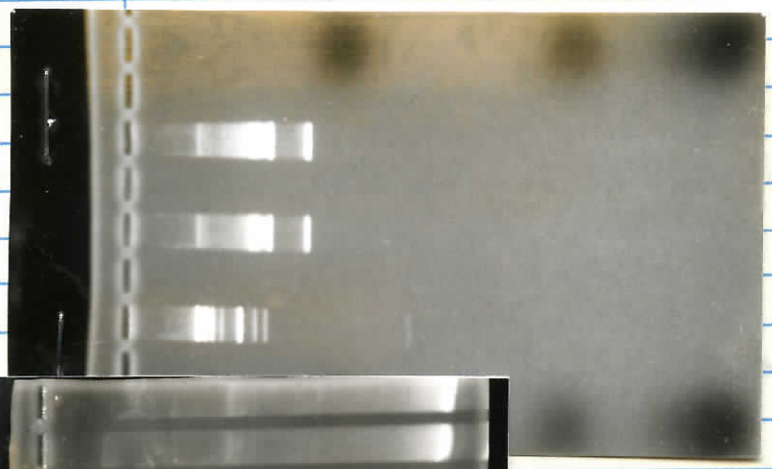
Dye
Water
Blue 148
Water
Dye
Water
Red 155
Water
Dye

On 4:10pm
Off Pause 7.45pm - 9.0pm
pm

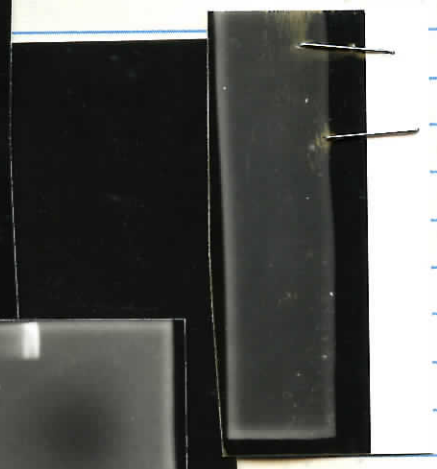
9.5
500mA V/cm

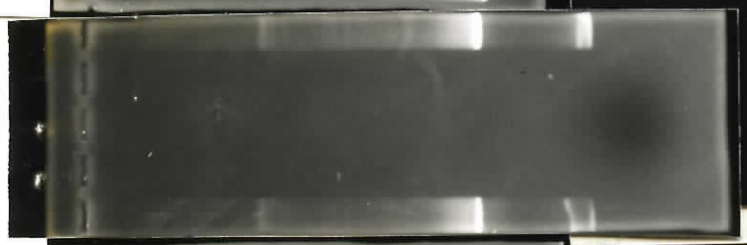
Should be ~10pm

Blues run until  — Scalpel



Conclusion: Run until Blue 2 is ~6cm
Cuts are fine (last of 55)
ssDNA is now well out of way.





Conclusion: Got over-run (fire alarm!!) but cuts pretty good.
Proceed.
+10% 8M NH4Ac
Melted 80-85° → *

Wed. Jan 19th

H [1.3] X prep. (contd.)

X/H [1.3] thind
thind [1.3] X/H

On 10.02am Off 11.15am

Dye
W
B'q² 148 X/H² Blue
W
B'q² 155 X/H² Red
W
Marker
W
Dye

~1.7% Seap. HX1

6 V/cm 100mA

Run until yellow dye is at wick (in this case 80 mm)

Blue 1 2 Yellow

Actual

On 12:40pm Off 3:40pm

~1.7% Seap 6V/cm 210mA

2nd Blue almost at wick

Dye
W
148 X/H B'q² Blue — same left
W
Dye
W
155 X/H B'q² Red — Nano left
W
W
Dye

B'11 148 Blue

B'11 155 Red

* ~1 ml ØOH / HOR
Cent¹ B'11 148 Blue
2X CIAA B'11 155 Red
Total vols. ~1.3-1.4 ml
Took
130 µl of each ✓
+390 µl EtOH ✓
2 back into 15 µl for last step
B'11 Cone 155 Red
B'11 Cone 144 Blue
Gel (over) →

Conclusion: Both are fine – as expected (λ is at highest conc).

Wed. Jan 19th

Subcloning Sal [5' flank] Eag

Cannot conveniently change Eag to Xho to use Bluescript since Sal & Xho are too close.
∴ subclone into BS & then change site. Paula needs this route anyway. Let her do it.
Later thought (Thurs) W131 pSP64 Sac → Xho can be used - it has a linker! third Pst (Sac) Acc Hinc 2 Xba Bam Ava Sma (Xho) Eco

Thurs. Jan 20th

On 10:58am Tests of [1.3] fragment

Off 11:35am 0.8% H x 4 Max

	Dye
	W
~12μl	Blue B'11 148 conc ≡ 120μl unconcentrated
"	Red B'11 155 conc ≡ 120μl "
	W
	Marker
	W
	Dye

Conclusion:
Φ Eag digest^n is fine.
Sal ~~[illegible]~~ of Kerin's work not complete (only 40%) on 2A.
continue digest^n:
- see B15 Sal/Xho^2 below

MX. 19am
Ok 12.15pm

	Dye
	W
2+1.5	Pst Eag
	Pst Sal
	Pst Sal
	Pst Xho
	Pst Xho
	W
	Markers
	W
	Dye

0.86 Hx4 max

Thurs. Jan 20th

Sal I [5' Blank] ~~Eag~~ → Xho

2B2 ϕ

Xho } 2A plasmid
Sal }

Take 3 μl 10x R1 salts
15 μl ddw
10 μl (2 μg) Paula's 2B2 B'3
2 μl Eag 1 Lot 21 (20u total)

B'15 2B2/Eag 0.07 μg/μl

37° 5.07 pm to 7.30 pm (frozen) — 2+15 B'15 Post Eag

Take 3.2 μl (4 μg) W131 #2A SHR 26 1.24 μg/μl
+6 μl 10x R1 salts
51 μl ddw
+ ½ μl (10u) Sal I Lot 47

B'15 2A Sal 0.04 μg/μl

37° 5.07 pm to 5.52 pm — 2+15 Post Sal Frozen

+ ½ μl (10u) Xho I Lot 26

B'15 2A Xho

+ ½ μl (10u) Lot 26 Xho

2+15 Frozen Post Xho

6.00 pm to 7:30 pm (frozen) — 2+15 Post Sal/Xho

+ ½ μl (10u) Sal I Lot 47

2+15 Post Xho/Sal

Fin. Pooled the Sal/Xho 2A & Xho/Sal 2A. Added equal vol. 1x R1 salts & added 1 μl more of Sal & Xho & back to 37° 12.55 pm to 3.45 pm Frozen

as B15 Sal/Xho 2

~0.04 μg/μl

(cont'd.) Two gel slices processed as usual 10% 8M NH_4Ac 75-85°C
ϕOH/HOQ, 2x CIAA.

Total vol ~450µl — 5' FLANK B'18 Probe — Red ← This was stained

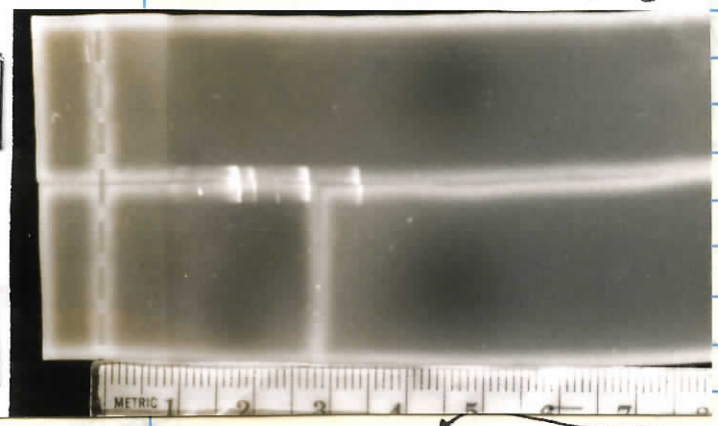
Total vol ~700µl — 5' FLANK B'18 FRAG — Blue ← This was not stained

45µl + 135µl EtOH → Back into 15µl 1x RI salts → 5 FLANK PROBE CONC Red

45µl + 135µl EtOH → 90µl RI salts + ½µl Xho I hot 2h 37° 12 min → 15µl → 5' FLANK Pre Xho Red; 5' FLANK Xho Red

70µl + 210µl EtOH → Back into 15µl 1XRI salts → 5' FLANK FRAG CONC Blue

Use these two for later test gel

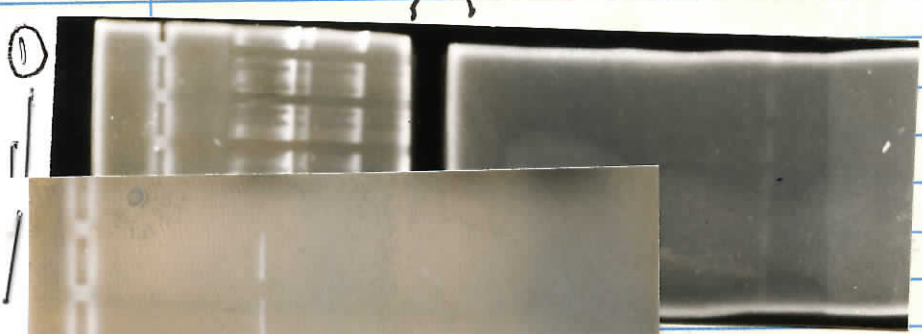


① B'17 Sal 5' flank Xho

Cut at 35 mm → 31 mm

Conclusion ①: Missed partly! Recovered stained slower rose the 20% B'17 probe

- can use it also to check Xho



② Conclusion: Repeat prep^n, but continue with this 5' flank fragment

Fri. Jan 21st

Sal [5' flank] ~~Eag~~ → Xho (contd.)

Take whole of B'15 2B2/Eag

✓ + 6µl (est. 3µg) B'5 OSEAG-XHO

✓ + 4µl 10× µ95 buffer

✓ + 4µl T4 Ligase

B'15 2B2 Eag Ligⁿ

2µl + 15µl

B'15 Pst Ligⁿ

15°C 10:40am to 11.25am

R.T. 11.25am to 12.05pm

37° 12.05pm to 1:15pm

2/3/86!

0.8% HX4 MOK

ON 12:26pm

off 1.13pm

(2)

Dye

W

40µl Blue 5' FLANK FRAG CONC

45µl Red 5' FLANK PROBE CONC

W

Marker

W

Dye

+ 4µl 8M NH_4Ac

+ 132µl EtOH

Back up into

54µl ddw

10× RS + 150mM NaCl

6µl

1µl Sal 1 (20v) Lot

37° 2.07pm to 3:47pm

Diluted with 30µl ddw

& applied to gel

B'17 2B2 Eag/Sal Xho

~0.05µg/µl

(1)

Dye

W

1

2

3

4

5

6

W

Dye

0.8% HX4 Seap

Not quite as much sample

0.8% Agarose HX4

ON 4:10pm

Off 9.10pm

~3V/cm

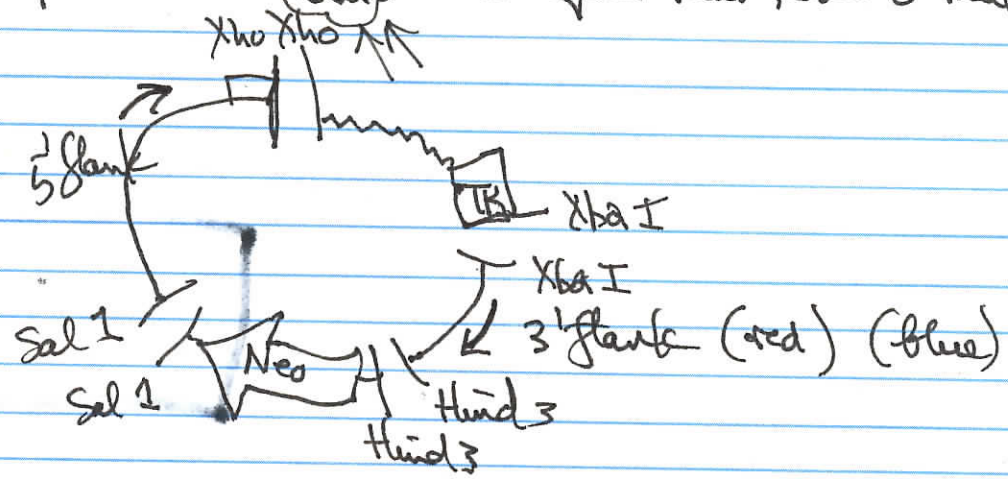
450mA

Mislabelled
as B'18 over page

Sunday, Jan. 23rd

ANPRADUP lig.

All (but one) fragments now available with ambiguity in 3' flank. ∴ assemble two ligations (Red & Blue) & proceed. (Error) Sal 1 ⟨Neo⟩ Hind, still to make.



Sal ⟨Neo⟩ Hind
~~Xho~~

Take 7.7 µl (10 µg) w125 #9 SHR34 Upcr 1.30 µg/µl B'19
10 µl 10x RI salb
82 µl ddw
1.5 µl Sal I (30 u) Lot 47

B'19
Neo
Sal/Hind

2 µl + 15 µl B'19 Pre

37° 2:25 pm to → 10 µl B'19 Sal Neo 7:00 pm

2 µl + 15 µl B'19 Post Sal

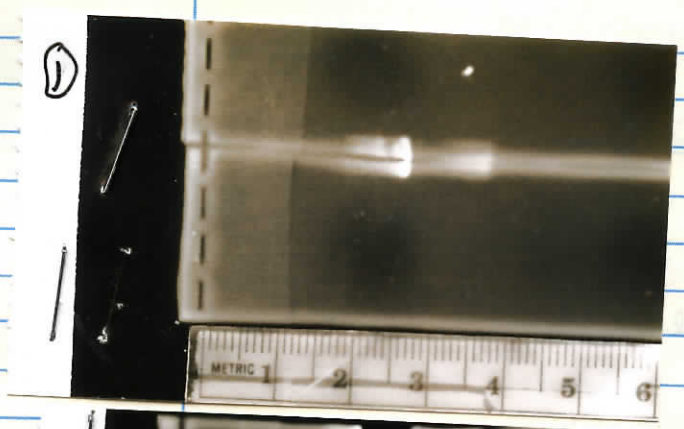
+1.5 µl (30 u) Hind 3 Lot 45

37° 2:25 to 5.50 pm

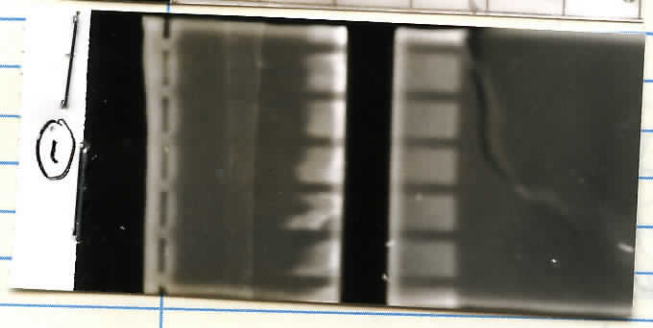
2 µl + 15 µl B'19 Post Sal/Hind

Use this only for probes

Repeat over.



22 mm
29 mm cut

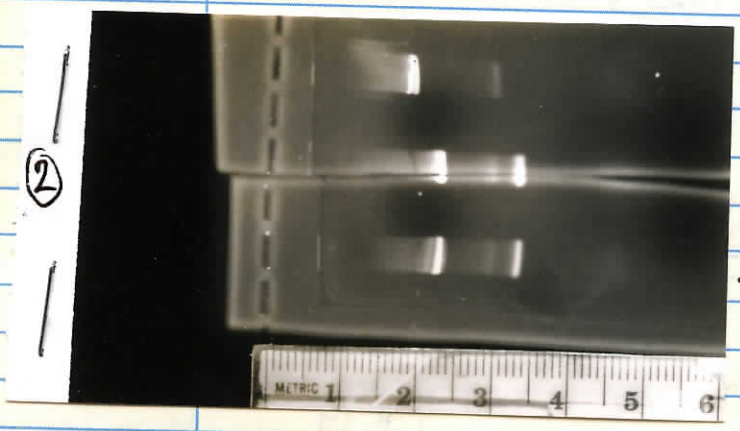


(1) Conclusion: Adequate (just) cut.
Proceed. → 870 mg + 90 µl 8M
80° Fx ØH/HOQ

B'20
pSP64 Sal/Xho

2xCIAA

Total vol ~800 µl



← Post Sal
← Post Sal/Hind
← Post Sal/Hind

(2) Conclusion
The digestion was fine: Sal OK, Hind OK.
Cuts at 40 mm } F B'20
35 mm
30 mm } M B'20
25 mm } S B'20

Processed to ØH/HOQ
2xCIAA.

75 µl of each plus 210 µl EtOH
→ back up into 15 µl for gel test (B'22)

B'20
Hind Neo Sal
M

Mon. Jan 20th

[illegible] pSP64 Sal / Xho

Hind / Neo / Sal frag^t

Take 7.7 µl (10 µg) w125 #9 SHR 34 V/ppcr 1.30 µg/µl B'19
10 µl 10 x R1 salts
82 µl ddw
1.5 µl Sal I (30 v) hot ⊂>

B'21 Neo Sal then Hind

37° 9:18 am to 11.20 am

2+15

B'21 Port Sal

+1.5 µl Hind 3 hot 45

37° 11:20 am to 1.50 pm

2+15

B'21 Port Sal Port Hind

50 µl + 150 µl ddw

Prep gel B'15 for pSP64 Sal / Xho²

0.8% Seap

pSP64 Sal / Xho² x 1/2

0.8% Ag cut 22 mm 29 mm

HK4

On 11.0 am

Off

3.5 V/cm

~~350~~ mA 300

Can use dye, since it is only vector, but don't need it.

Take 50 µl B'15 Sal / Xho² + 50 µl ddw. ~~add~~

2+15 B'21 Port Sal

2+15 B'21 Port Sal Port Hind

Dye

0.8% Seap

B'21 Neo Sal Hind + 4

0.8% Ag

HK4

On 2:56 pm

Off ~ 5.0 pm

<3 V/cm

450 mA

(1)

on 10.35am
off 11.45am

0.8%
Hx4
max

(1) Byf
W
2+13 { Pre B'23 lig^n
Post B'23 lig^n
W
~~75 pl~~ 15 { B'20 F
B'20 M
B'20 S
W
Marker
W
Byf

(5X)

(1) Conclusion: B'20 M is good — save.
lig^n: worked — ↓ vector & ↑ frag^t or repeats!

(2) see over

(2)

	Last trip
pH5α 1/7	~180 pl
~35 pl Rest of	B'23 lig^n
0°	11.26^am to 11.58
42°	90 sec
	Plated 37° 12.15pm
	10am
	Stationary 37°

14 colonies or "haloes"
Mins attempted 10.25am Sat 5:25pm

(2) Conclusion: Not this time!

Mon. Jan 24th

Subcloning of Sal [5' flank] Xho in pSP64

Rough attempt without gels.

Take ✓ 210 µl 5' FLANK B'18 FRAG (Blue)
✓ + 40 µl B'20 pSP64 Sal/Xho
✓ + ~750 µl EtOH
Back up into 10 µl 1x p95 ligⁿ buffer

B'23 pSP5'FLANK LIGⁿ

15° 5.11 pm to 6.04 pm
R.T 6.05 pm to 6:35 pm
37° 6:35 pm to 6:45 pm
R.T 6:45 pm to ~8.30 pm

2+13 PRE B'23

2+13 POST B'23

cold room overnight

Transformation B'23 pSP64/5'FLANK

B'23	DH5α 12/6	80 µl	Ø25 DH5α B'23 O.N.	40 µl
	B'23 ligⁿ	2 µl		2 µl
	0°	8.35 pm to 9.18 pm	~11am 0°	~12 pm
	42°	90 sec	≤ 2°	~90 sec
	Plated	Rest 20, 5, 20/16, 5/16		Thes 37° 12:15 pm to Thes

9.30 pm 37° 9.30 pm

9.30am looks like 0
Repeat with better cells.

Catch up gel

Sat 10

Sun Jan 30th

	Dye W	Transformation	B'24 Blue	B'24 Red	B'24² Blue	B'24² Red
	Marker W					
2 + 3	Pre B'25 Blue	DH5α 1/7	~100 µl	~90 µl	120 µl	120 µl
	Post B'25 Blue	hig^n	1 µl B'25 Blue hig^n	1 µl B'25 Red hig^n	1 µl Blue hig^n	1 µl Red hig^n
1	Pre B'25 Red					
	Post B'25 Red					
	W					

0° 11.01 am to 11.34 am ~5.29 pm

90 sec 42° ✓

Plated Rest — Red B'24

20, 5, 20/16, 5/16 — Blue B'24

11.54 am Sun to 37° → Man. (go to B'29)

B'22 mins ⟷ see over

0.8% HX4 Max ~11 am to ~12 pm

1

Conclusion: Quite good proportions; doesn't need quite so much B'11/155 Red. Ligations are promising.

Size sh'd be ~14 kb

1.0	Neo
1.3	3' flank
4.7	TK Vector
6.4	5' flank
13.5	

DHS 1/7

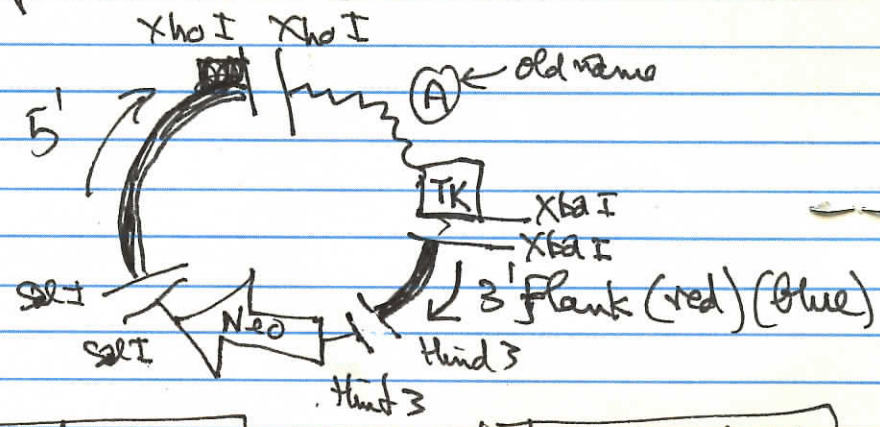
Natriuretic peptide receptor 1 (old name ANPRA)

Fri. Jan 28th

First attempts at making complete construct for NPR1 duplication (see B'19)

Fragments are now available:—

B'24² Plated 15µl plate 6:?pm 1st
NZY 1 Amp
37°
N6:15pm
9.15am



B'25 Blue lig^n

B'25 Red lig^n

Take ✓ 20 µl A'7 Xho TK Xba M
60 µl B'20 Hind Neo Sal M
✓ 320 µl B'18 5' FLANK FRAG (Blue) whole of sample

120 µl total 3 Flank Blue: 100 100 µl; 60 60 B'11 / 148 Blue; 480 480 Etoh (3 vols)

240 µl total 3' flank Red: 100 100 (→80 µl in fact); 120 + 120 µl B'11 / 55 Red; 660 660 + µl Etoh

10 µl 1 x p95

2 µl + 13 µl PRE Blue B'15 + 1 µl T4 ligase

10 µl 1 x p95

2 µl + 13 µl PRE Red B'25 + 1 µl T4 ligase

15° 5.52pm to 6:28pm
RT 6:29 to 6:33

Important Frozen Warm Sun

To cold

10.15am Sun

2 µl + 13 POST Blue B'25

2 µl + 13 POST Red B'25

Oligo # 07236 5' note: OSECOBAM

Date made: [redacted]

LENGTH: 12

% G+C: 50%

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

500 Å 0.2 uM NAC-020-5 Prime Synt 0 0 4

Oligo # 5' note:

```
10:01:49A , 1/29/94
Run ID                    - 394 Synthesis Setup Listing -        (Version 2.0
Column 1
                                                  1:53:40P , 1/29/94
Run ID  :
Cycle   : 02UM USER
End Proc: End CESS      (DMT = Off)
Sequence: 07236

Total bases =  12
        A=  3, G=  3, C=  3, T=  3, 5=  0, 6=  0, 7=  0, 8=  0
        (mixed bases=  0)

MW:   3643.4

     5'> AAT TGG GAT CCC <3'
```

Sat. Jan 29 th

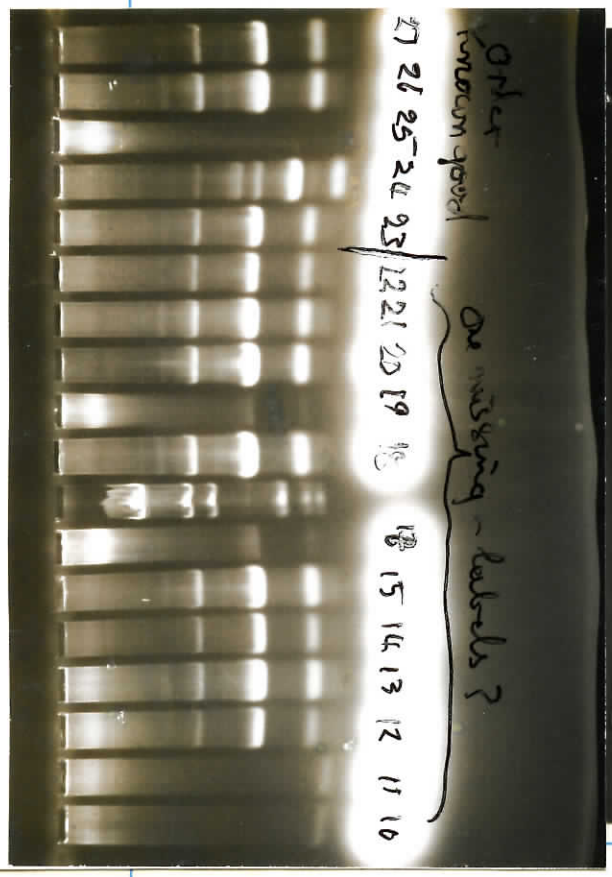
Eco R1 → Bam H1

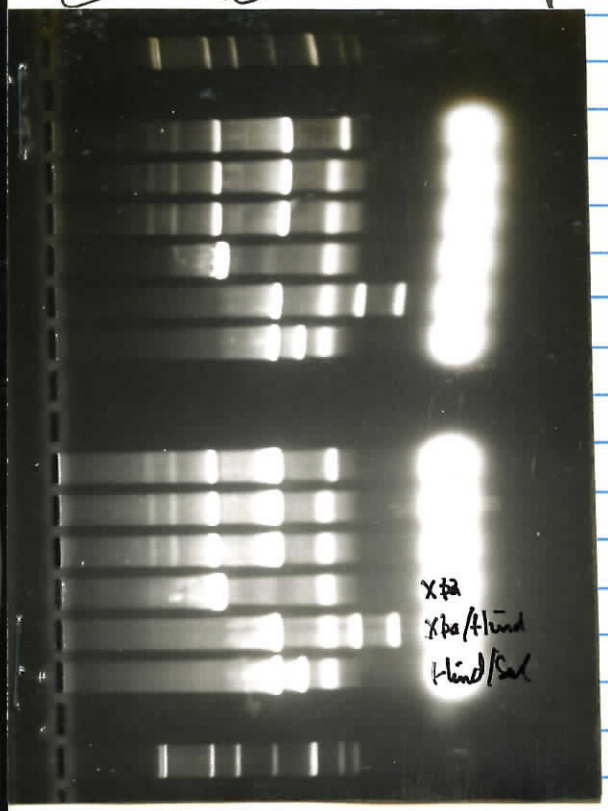
G AATT GGGATCCC AATTC

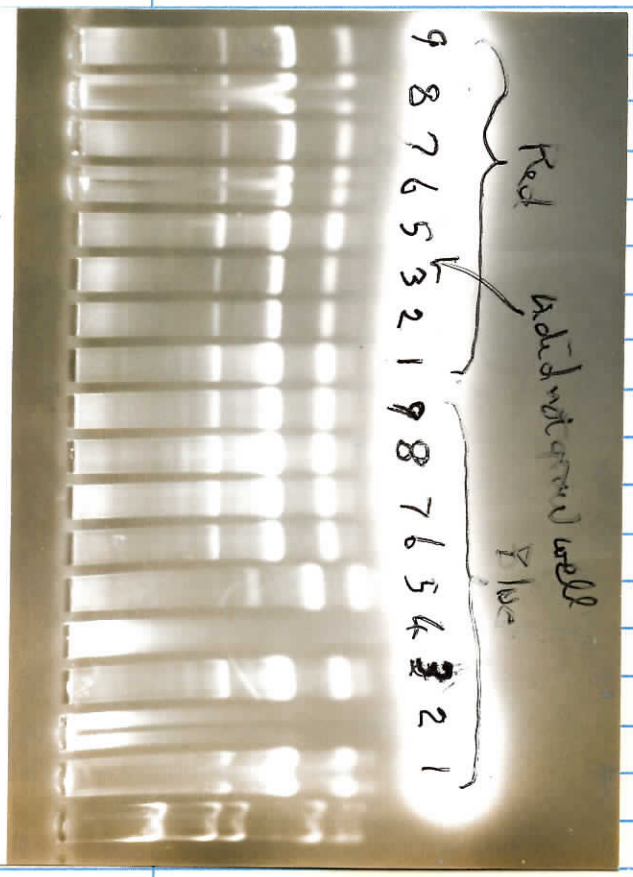
Ex Eco Bam H1 Ex Eco

Back up into 500 µl of TE 1XCIAA
& stored as B'26 OSECOBAM

B'24 8 Blue | B'24 26 Red | streaked out on Wed.







Conclusion: Missed!

Xba linearized to ~9 kb

Xba/Hind → 1.3 kb + 1.8

Hind/Sal → 4.0 + 3.2

So it has 1 Xba site
2 Hind sites
1 Sal site

Xho not there so the site change is just gone now!

Design Errors

Sal will ligate into Xho

on 3:10 pm off 4:40 pm

0.8% MX 4

Wed. ~~Feb~~ 2nd

1st clonings of NPR1DUP

Sylvia picked the very dispersed, satellite-rich, colonies & streaked out 3 mate minis. Some labelling problems of gels but clear result

6/9 Blue look good; 18/25 Red look good.

Proceed with 8 Blue and 26 Red

KOAc supps. + 350 µl isoprop.

Ppts. washed. Back into 50 µl .001M EDTA as

B'24 8 Blue	B'24 26 Red

Bye
W
Marker
W
Uncut
Xho
X/Sal
X/Xba
X/Xba/Hind
X/Hind/Sal
W
W
Uncut
Xho
X/Sal
X/Xba
X/Xba/Hind
X/Hind/Sal
W
Marker
W
Bye

Take ✓ 32 µl 10×R1 salts
✓ + 272 µl ddw
✓ + 16 µl B'24 8 Blue
✓ + 4 µl (5×160) Xho1 lot 26
40 µl ✓ → 8 Blue Uncut

✓ 32 µl 10×R1 salts
✓ + 272 µl ddw
✓ + 16 µl B'24 26 Red
✓ + 4 µl Xho1
40 µl ✓ → 26 Red Uncut

✓✓✓✓ 40 µl

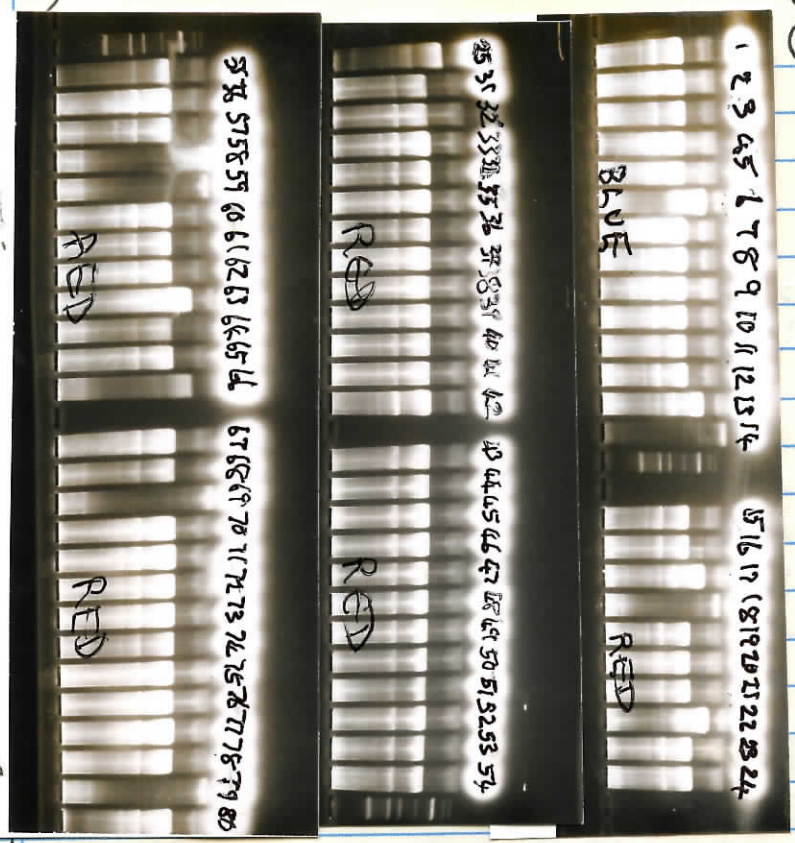
Rest
+ ½ µl Sal I lot 47 → 8 Blue Xho
+ ½ µl Sal I → 8 Blue X/Sal
+ ½ µl Xba I lot 29 → 8 Blue X/Xba
+ ½ µl Xba I + ½ µl Hind 3 → 8 Blue X/Xba/Hind
+ ½ µl Hind 3 lot 45 + ½ µl Sal 1 → 8 Blue X/Hind/Sal

✓✓✓ 40 µl

32° 12:15 pm

2:30 pm

Rest
+ ½ µl Sal I → 26 Red Xho
+ ½ µl Sal I → 26 Red X/Sal
+ ½ µl Xba I → 26 Red X/Xba
+ ½ µl Xba + ½ µl Hind → 26 Red X/Xba/Hind
+ ½ µl Hind + ½ µl Sal → 26 Red X/Hind/Sal



(3) on 2:02pm off

Dye	
Mark	
55	
56	
57	
58	
59	
60	
61	
62	
63	
64	
65	
66	
Wat	
67	
68	
69	
70	
71	
72	
73	
74	
75	
76	
77	
78	
79	
80	
Dye	

(2) on 12:50pm ~~off 1:32pm~~

Dye	
W	
25	
31	
32	
33	
34	
35	
36	
37	
38	
39	
40	
41	
42	
W ~~43~~	
44	
45	
46	
~~47~~	
~~48~~	
49	
50	
51	
52	
53	
54	
[redacted]	
Marker	
Dye	

(1) on 11:42am ~~off 12:36pm~~

Dye	
W	
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	
11	
12	
13	
14	
Marker	
Water	
[redacted]	
15	
16	
17	
18	
19	
20	
21	
22	
23	
24	
W	
Dye	

0.8% 1X4 Max

Thurs. Feb 3rd

B'24² minis

Picked early & still not v. satellite except #6 & #7, 14 & B'24² BLUE colonies (#14 not streaked). To 37° at 10:25am

B'24² RED #15–25 ↗

and #31 — 55 (no 26–30)

RED → 37° 11.25am

B'24² Blue

RED #56 — #80 (only ~6 more left)

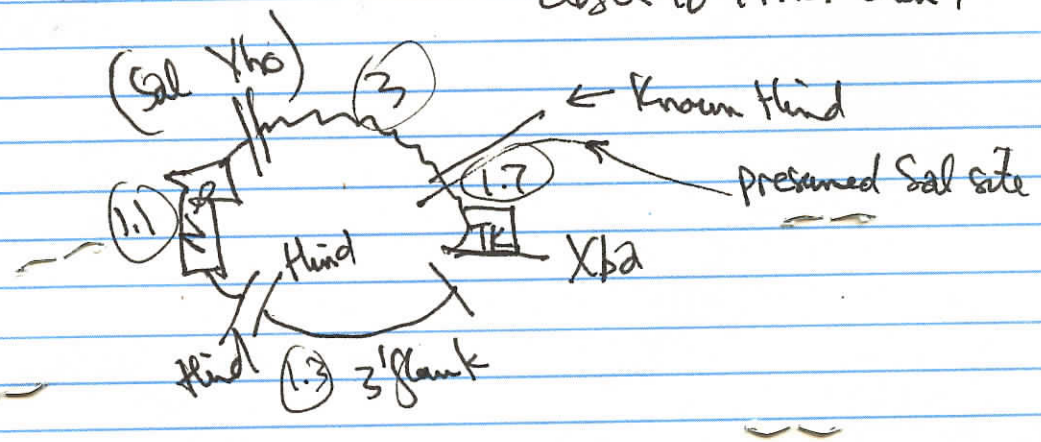
37° 11.55am

(1) Conclusion: None of Blues are correct or 15–24 of Reds. Finish the other two gels. If any Red is correct repeat Blue. If none – proceed with correction strategy. Probably these minis were a waste of effort because conc.ⁿ of 5'flank Sal–Xho is low & is ↓ to 50% by orientation AND can self ligate.

(2) & (3) — confirm the problem

Glorious yield of the 3 fragment construct: probably closer to 7.1kb than 9

B'24² Red



(B) OS

Dye
Mar
55
56
57
58
59
60
61
62
63
64
65
66
W
6
6

into 500 µl TE 1XCIAA
Assume 0.5 µg/µl

B'32 OS
XhoI
↓XhoI NotI XhoI

07247
de: 2-3-94
II: 12
: 83%

5' note: OS XhoNotXho

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

NAC-020-500

Run ID :
Cycle : 02UM USER
End Proc: End CESS (DMT = Off)
Sequence: 07247

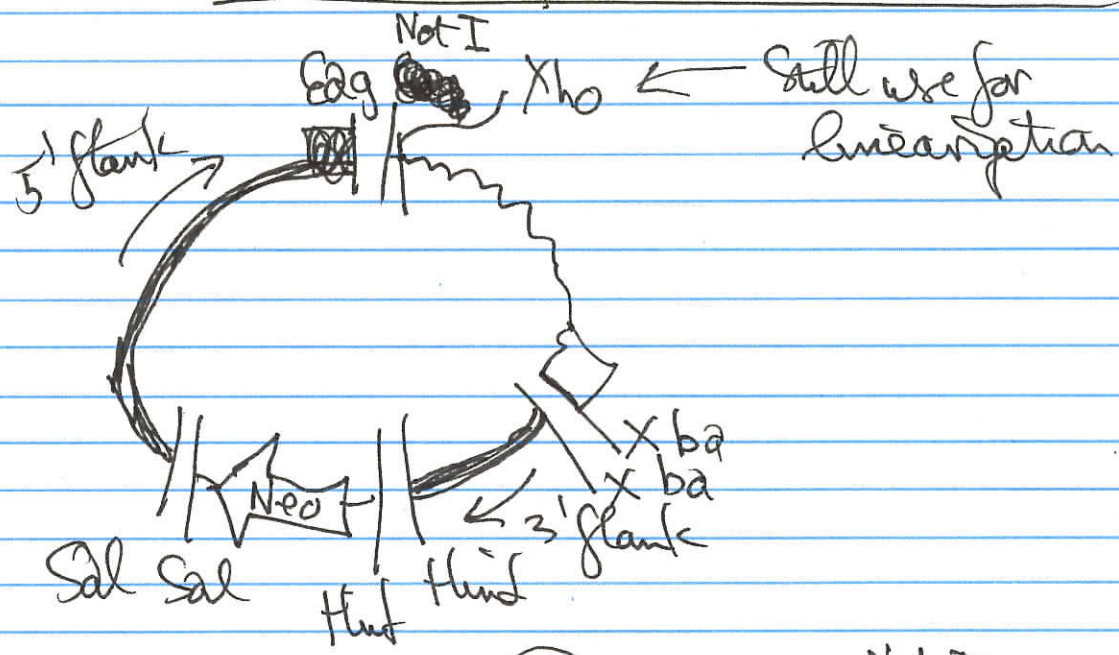
Total bases = 12
A= 1, G= 5, C= 5, T= 1, 5= 0, 6= 0, 7= 0, 8= 0
(mixed bases= 0)

MW: 3643.4

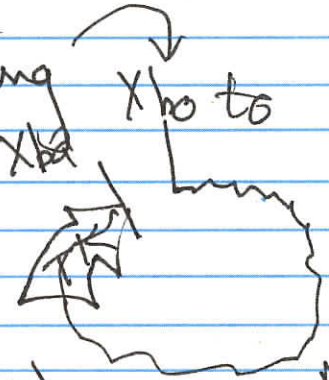
5'> TCG AGC GGC CGC <3'

Thurs. Feb 3rd

Correction of NPR1 DUP design

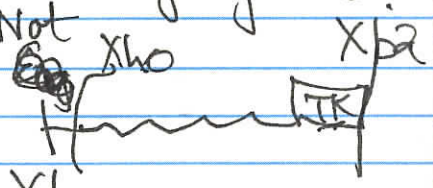


Requires changing Xho to Xba



Xho — Not I — Xho
cutting with Not I
Xba & Not I for fragment

(There are 2 Eag I sites in pMCI TK but no Not I. Two have same overlap)



OS Xho Not Xho ≡ Xho Eag Xho

CTCGAGCGGCCGCTCGAG

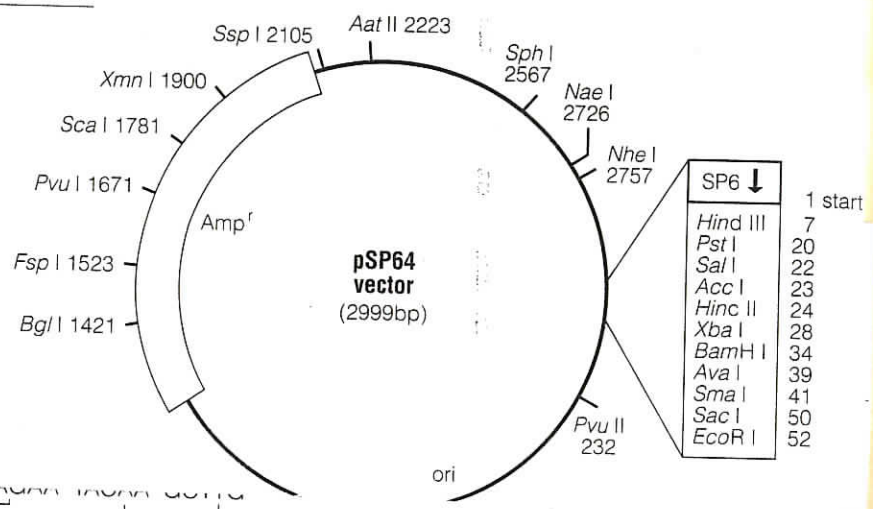
GAGCCGCGGCTCGAG

XhoI — Not I — Eag I — XhoI

(Cont'd. →)

pSP64 Vector

Product	Size	Cat.#
pSP64 Vector	20µg	P1091



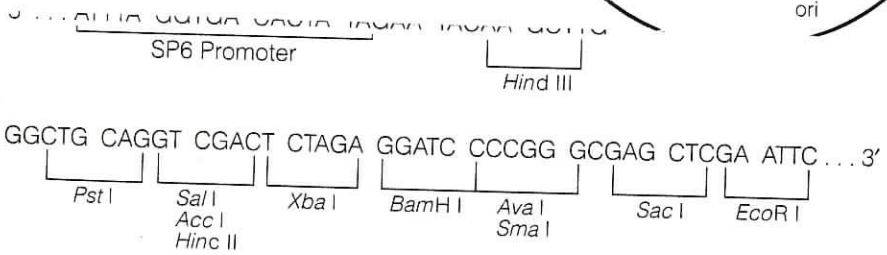


Figure 3ZZ. pSP64 vector promoter and multiple cloning site sequence. Base #1 is the transcription start site for SP6 RNA polymerase. The sequence shown corresponds to RNA synthesized by SP6 RNA polymerase.

Use our telephone numbers below.

To order:
800-356-9526
608-274-4330

Technical Services:
800-356-9526

Fri - Feb 4th

~~XhoNot~~ Xho ← ~~Xho~~ [TK] Xba

Sylvie had digested w149 #13 with XhoI on SH R37 at conc^n of 0.1µg/µl
I label this tube B'35

✓ Take 10µl B'35 w149 #13 XhoI (1µg)
✓ + 1µl 10x PBS.
✓ 85-90° 5 min [B'35 13 XhoHeat]

whole
✓ +2µl (~1µg) B'32 OS XhoI → XhoI NotI, XhoI
✓ +2µl 10x p95 lig^n buffer
✓ +5µl ddw
✓ +1µl T4 ligase 15° 3:28 pm to 4:03 pm
RT 4.02 to 4:32 pm
37° 4:32 to 5:09 pm

[B'35 13 Xho LIG^N]

2+15 [B'35 13 XhoLIG^N POST]

Rest to cold mon
Frozen

✓ 10µl (~0.05µg) [B'35 #13 XhoLig^n Diluted]
✓ +80µl ddw
✓ +10µl 10x PBS
✓ 85° ~3 min → to ice

Rest Frozen

[B'35 TK]

Transformation		
B'35 DH5α 1/7	80µl	
B'35 #13 Xho diluted	10µl	
0°	5:17 pm to	5:46 pm
	42° 90 sec	
Plated	Rest 20	5 20/16 5/16 37°
		1X Amp

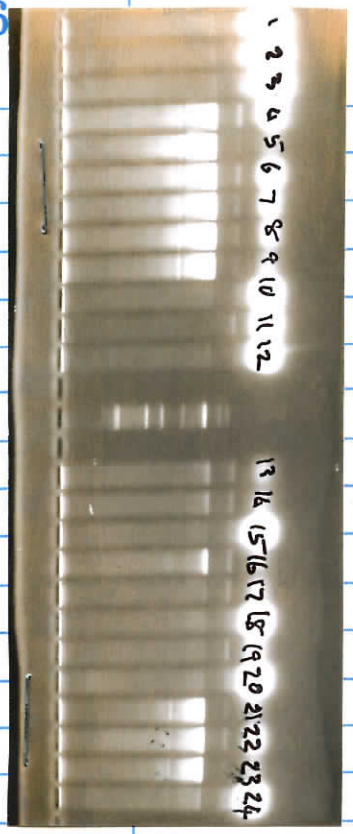
Rest goes here

10:50 am

5:57 pm

[B'34 TK mono]
1-24
37° 11:11 am
Cat

(B)



Conclusion The ones with the heavy precipitate were, in general, the poorer ones! But in any case none have become Not I.

Re-inspect oligo & protocol.

Better check post-ligation from B'35.

On 12:30pm
Off 1:42pm
Max 0.8%
HX4

Day
W

Mon. Feb 7th

B' 35 mins

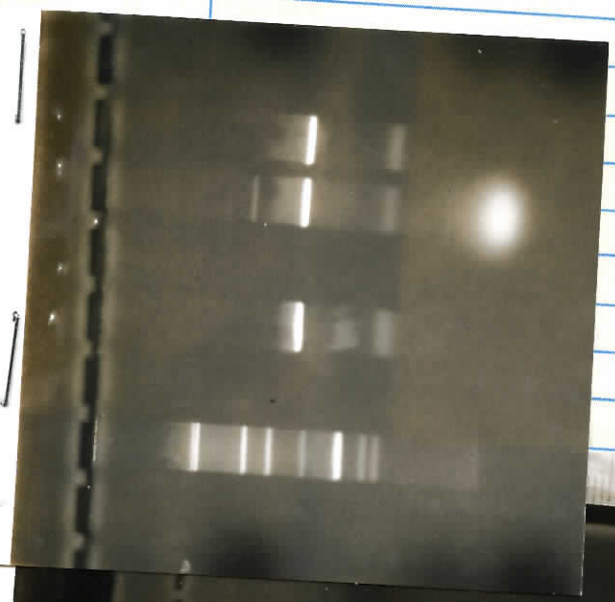
Processed to KOAc Sun ~10am (23 hr.). left until Mon. prior to cent. As usual thereafter – but very low or no ppts. in many tubes. Very odd.

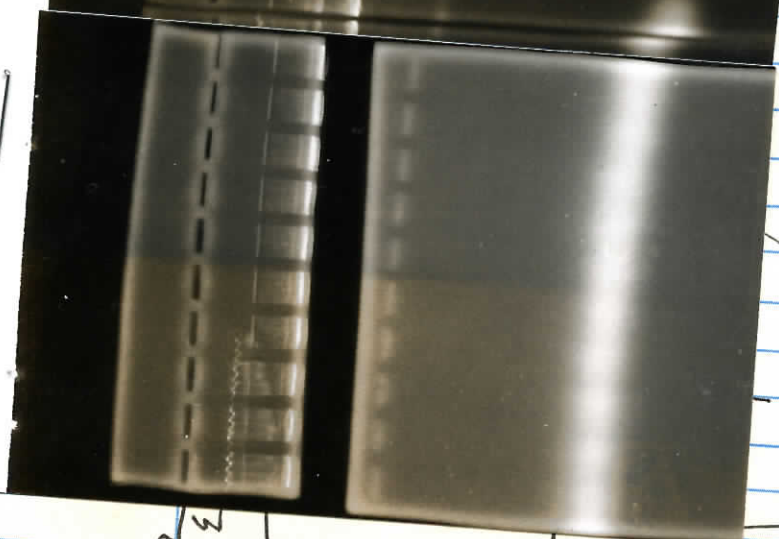
Take 52 µl (2×26) 10×R1 salts
+ 464 µl ddw
+ 8 µl (26×3 units) Not I Lot 714 (10 µl)
520 µl

B' 35

20 µl - - - each + 1.5 µl of minis #1–#24
37° 10:51 am to 12:11 pm

Marker
W





Conclusion:
No obvious reason for failure — fig. gave some [illegible] if not ∴ repeat!
∂ use φOH/HOQ for Xho digest? May need to prepare fragments to ↓ ssDNA ∂ remove [illegible] ∂ trim

This was the trouble

W

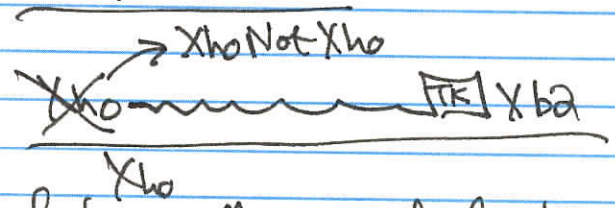
Bye

B38 E
B38 M
B38 S

fragments made. 0.8M NH_4Ac ~ 85°
1x φOH/HOQ
2x CIAA

100 µl of each + 300 µl EtOH
Back up into 20 µl 0.001M EDTA
as B38 F Conc | B38 M Conc | B38 S Conc

Man. Fab 7 ul



Repeat of ligat^n & gel check

Take ✓36.4 µl ddw
(≈5 µg) → ✓7.6 µl w149#13 super mini CSHR23 0.66 µg/µl
Now labelled B'39
✓5 µl 10xR1 salts
✓1 µl (20u) Xho I lot 26

37° 3.25pm to 4:30
37° still

B'39
13 Xho

1µl + 16µl
B'39 Post

On 4:40pm
off 5:20pm
0.8%
1x4 Max

	Agr
	W
1+16	B'39 13 Xho (Post)
1+16	B'35 w149 #13 Xho I (ex Sylvia)
	W
2+15	B'35 13 Xho Lig^n (Post)
	W
	Marker
	W
	Agr

Switched

5.55pm 1xHOQ/HOQ 2xCIAA

B'39
13 Xho
Post ϕ

Take (≈2µg) ✓20 µl B'39 13 Xho Post ϕ
✓+2 µl 8M NH$_4$Ac
+66 µl EtOH

Back up into 14 µl ddw
✓2 µl 10x p95 lig^n buffer
✓4 µl B3209 X→XNX
✓+1 µl T4 ligase

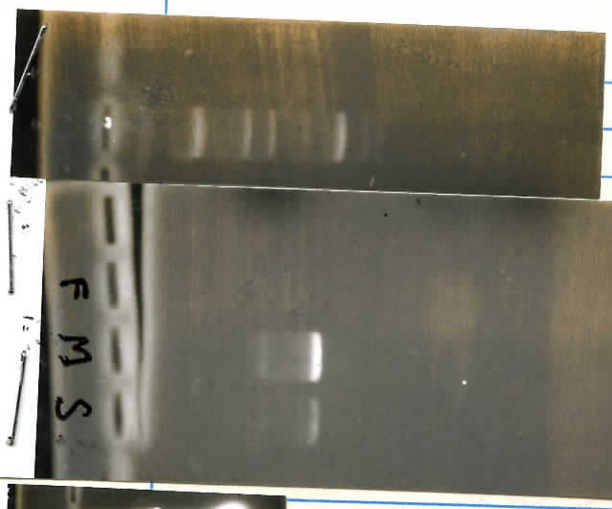
B'39
Lig^N

15° 6:46pm to 6:56
Cold 6:56pm to 9:32am R.T 9:32 to 10:02 37° 10:02am to 10:17

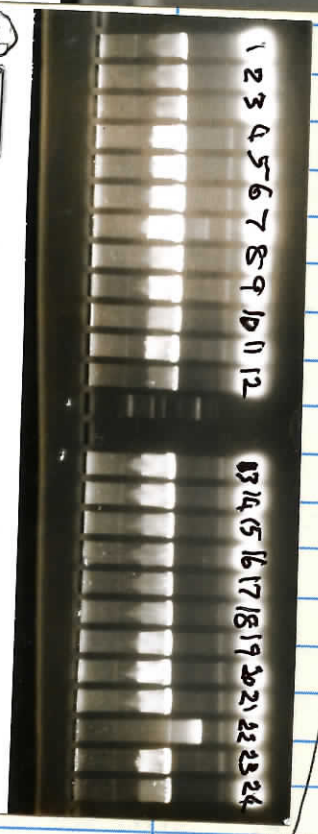
0.8% E

(3)

(1)



(2)



(2)

Dye
W
1
2
3
4
5
6
7
8
9
10
11
12
Mark
Water
13
14
15
16
17
18
19
20
21
22
23
24
W
Dye

Conclusion (2) 24/25 are correct, now!

Streaked out #11

as B'38 TK Xho Not Xho 3:45pm Thurs

11

Pooled 4, 5, 6, 8, 9, 13, 14, 20 & 21

to get ~230µl

+ 2µl (40u) Xba I and to 37° 3:40 pm to

i.e. 3.5u/20µl

B'40 Xba pool

Also 10µl of #22 + 10µl of Xba pool

B'40 Xba control

Also 10µl of #22 + 10µl #23

B'40 No Xba control

Extract frozen

5:43 pm Frozen

0.8% EtX4 max
~~On 1:27pm~~
~~off 2:38pm~~

Tues. Feb. 8th

Xho Not Xho

~~Xho~~ ~~TK~~ Xba (contd.)

~~On 7.42pm~~ ~~off~~

0.8% EtX4 ~ max

(1) Dye
W
F } Conc
M } B'38
S
W
W
Dye

(1) Conclusion: Proceed with M.

		B'38 M	B'38
DH5α B'38 (fresh)	80 µl	80 µl	80 µl
DNA	5 µl F conc	5 µl M conc	5 µl S conc
0°		7:21pm to	7:19pm
42°		90 sec	

Mini B'38
M #1–24
32° 10:52am to 10.15pm
stationary 37°

Processed Thurs. am. as usual

Take 104 µl (26×4) µl 10×RS Alabet
920 µl ddw +1.5M NaCl
16 µl (26×6 u) Not I Alabet (10u/µl) M & S
To 40 µl, each +3 µl minus B'38
37° 12:08pm to 1:39pm & back to 37°

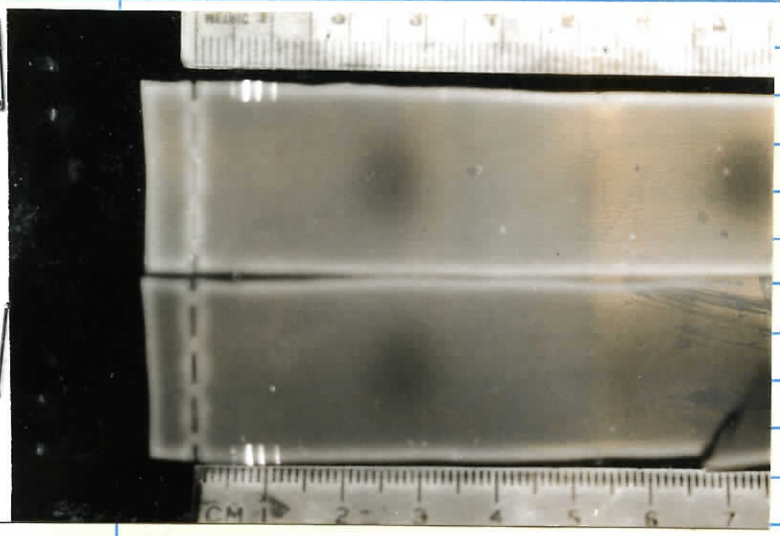
5/16 have many
5/16 have some

Can use excess for Xba I & prep. gel.

B(4) Not 1→24
30/40 µl

(B)

(1)



Wed. Feb 9th

· Sal [5' Blank] Eag

Take ✓ 3 µl 10×R1 salts
✗ + 16 µl ddw
✗ + 10 µl (2 µg) Paula's 2B2 B'3
✓ + 1 µl (10u) Eag 1 Lot 29
✓ + ½ µl (10u) Sal 1 Lot 47

2 µl } B'43²
1 µl } 37° 7 pm
Fri 10.10 pm

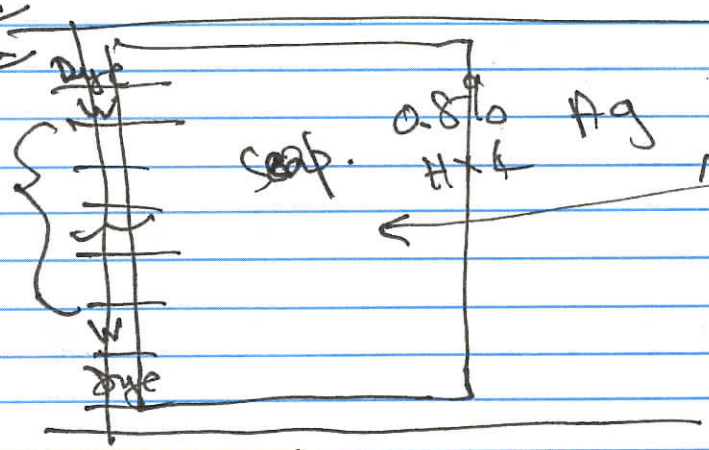
37° 11.19 am to 12:50 pm + 60 µl ddw & to gel

B'43
2B2
Sal/Eag

NO.8 µg/µl

M 1:05 pm
~~off 3:45 pm~~

Expect 6 or 7 total slots



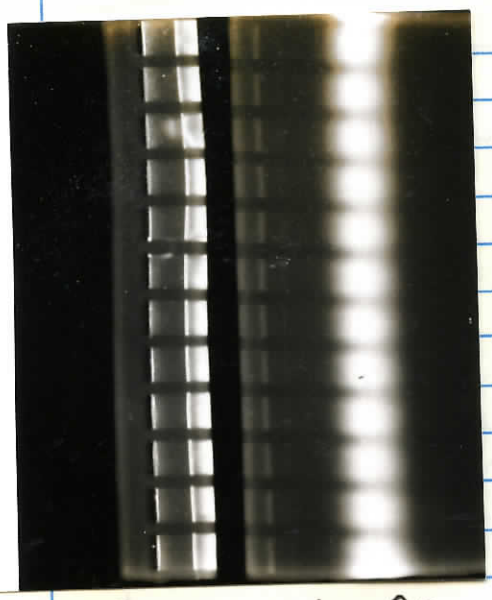
① Conclusion: Forgot that Sal I didn't cut well (see B'15 plasmid expt.). Repeat with 2× more of each enzyme & digest for several hours. Use R1 10×
Or, is it the mig.?
For safety cut out the 9mm–11mm band

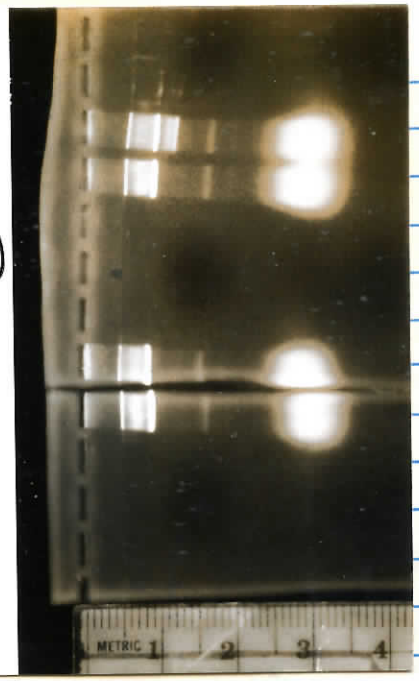
② Conclusion: good. M is fake!! & F is usable!

Dye
W
F
M
S
W
Marker
W
Dye

B'43	F
B'43	M
B'43	S

M 8:07 pm ~~off 9.55 pm~~
0.8% HX4 2 MAX
73



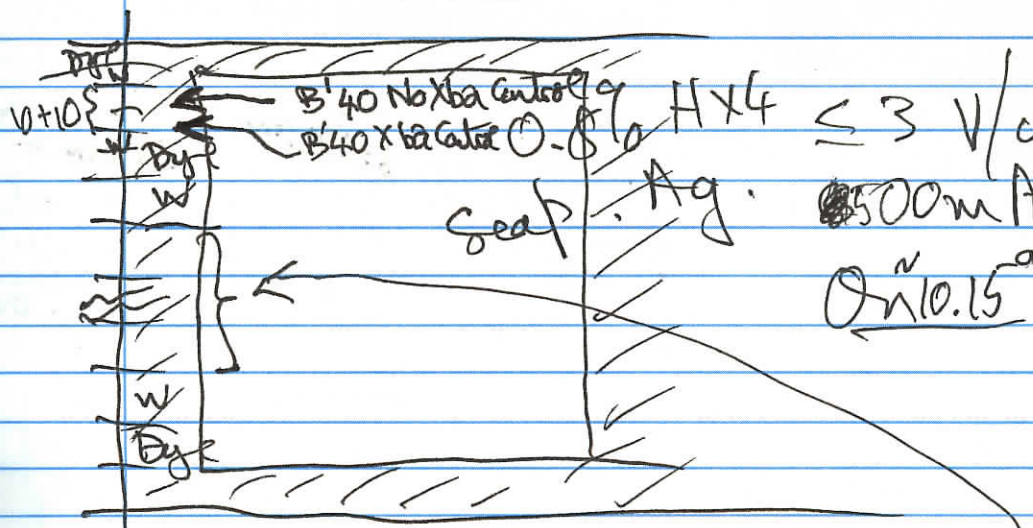


Conclusion (1) Xba worked fine
judged by NoXba & Xba controls
Fragments prepared

Conclusion (2) S[redacted] is terrific

Fri. Feb 11th Ice storm day!

Not Xba ~~~~~ TK Xba



≤ 3 V/cm

500mA

On 10:15am Off 12:15pm

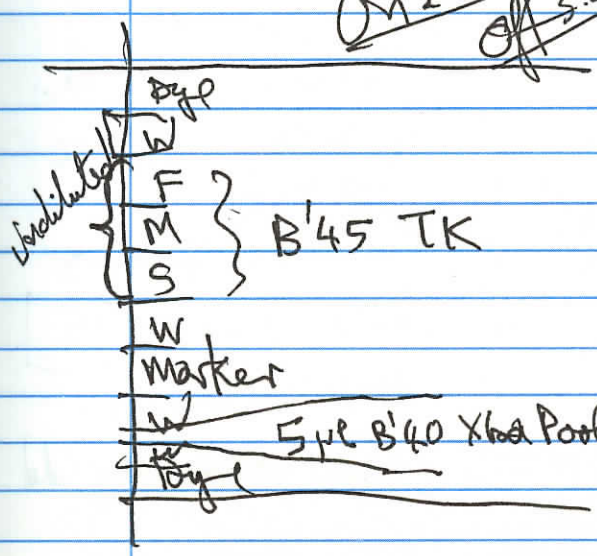
100µl of B'40 Xba pool + 100µl ddw

B'45 TK F
B'45 TK M
B'45 TK S

B'45 fragments made & processed to the 1x HOQ/DOH, 2x CIAA stages.

On 2:39pm Off 3:22pm

0.8% HX4 Max



5µl B'40 Xba Pool + 15µl ddw

	B'46²	B'46 Blue	B'46 Red	
(Fresh) Cell DH5α 1/7	230 µl	80 µl	230 µl	80 µl
DNA	Rest of lig	2 µl B'47 Blue lig	2 µl B'47 Red lig	Rest of lig
	1:35 pm – 2:05 pm	Sat Fri 2 0° 6:03 pm	to 6:33 pm	1:35 pm – 2:16 pm
	90 sec. 42°			Not accurate time at 42
	Plated			
	Rest 20	5, 20/16	5/16	

NZY 1X Amp
37° 7 pm Fri

Conclusion ① ligations worked (or there's a DNA's!). Proportion of TK c'd be 2X and of 5' Blank c'd be 4X.

Next time out 37° w/ less enzyme. 3+e off

Streaked out as B'46 BLUE 1

See also B'59

Processed Sun 2X to 100 µl
Gel B'48

Plated on 10 plates each 37° 2:30 pm Sat

9:15 am Sat
1 Blue ! Processed.
Run LIG.N gel. Set up a new larger set of transformations & proceed.

(2) Red ← still zero
(2) Blue ← 4 more

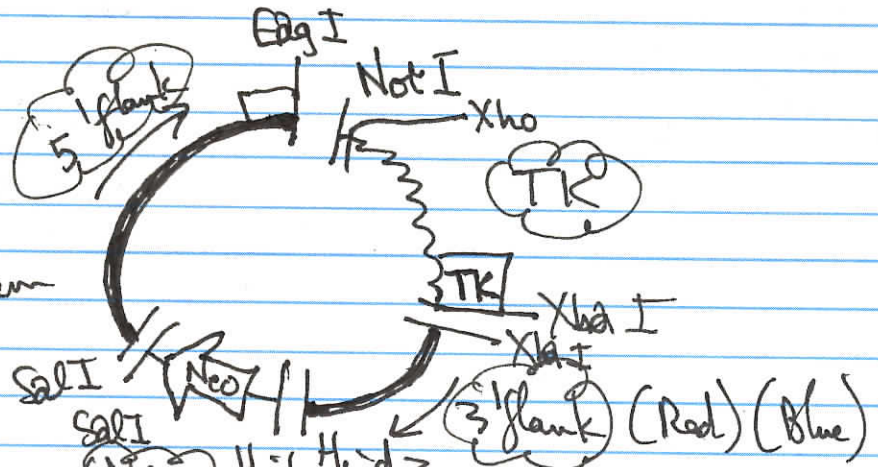
B'46 BLUE 2 – 5

10:15 am Sun
6 pm Cold

Processed Mon.
Gel B'48
Nice normal pots.!

Fri. Feb 11th

Second attempt at NPR1DUP construct



0.86 9HX4 Mix
9.58am
11.15am

Byp	1
W	
Pre	BLUE
Post	
Pre	RED
Post	
W	
Marker	
W	
Bye	

2 + 13

B'47 Blue hig^n

B'47 Red hig^n

2 µl S
B'45 (Not TKXba)

60 µl
B'20 (Hind Nes Sal M)

75 µl (~2/3 total)
B'43 M
(Sal 5' flank Eag)

68 µl

Rest

100 µl

B'11/148 Blue
(3' flank)

150 µl B'11/155
(3' flank) Red

504 µl

EtOH

657 µl

Back into 10 µl 1x p95 hig^n buffer

2+13 µl Pre Blue B'47

+ 1 µl T4 ligase

2+13 µl Pre Red B'47

+1 µl

To 4°C

15° 5:02 to 5:32
RT 5:32 to 5:47
37° 5:47 to 6:02

Rest to cold

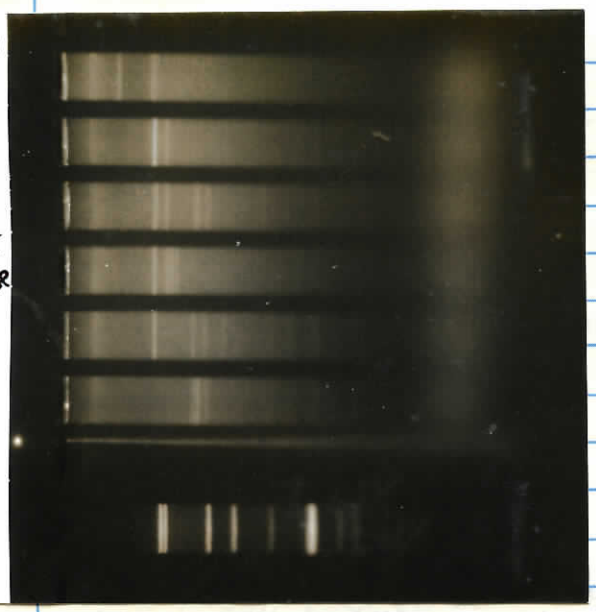
2µl+13
POST Blue B'47

B'47 Blue hig^n

B'47 Red hig^n

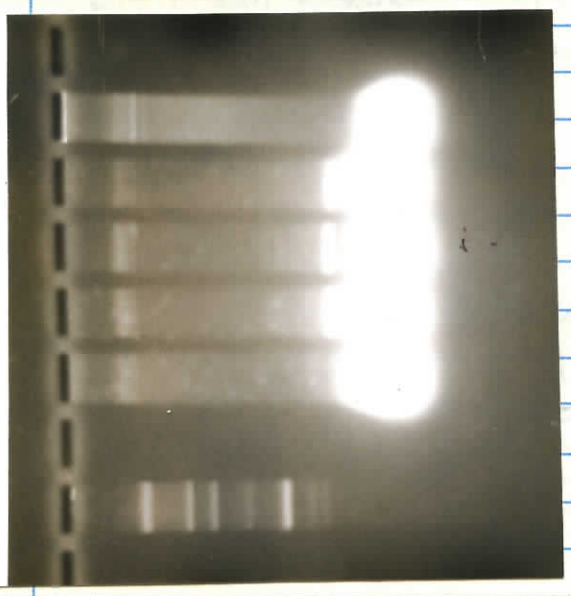
2+13 µl
POST Red B'47

(1)



Post RNAse

conclusion (1): Very odd! whatever it is, it has an Xho site
[redacted] a Sal site
an Xba site
& several(?) fund.
Just repeat digests with more DNA & more time



Conclusion (2): Still no good. Let's try the new ligation & re-make 5' flank for next try.

Sun. Feb. 13th

B'46 Blue 1 test

Take 14 µl (7×2) 10×R1
115 µl ddw
11 µl B'46 Blue 1
+1.5 µl (50u×6) Xho 1 Lot 26
5× 20 µl

20 µl → B'49/1 No Enz

½ µl Sal 47 → B'49 1 Xho

½ µl Xba 49 → B'49/1 X/Sal

½ µl Xba ½ µl Hind 45 → B'49/1 X/Xba

½ µl Hind ½ µl Sal → B'49/1 X/Hind/Sal

37° 11.10am 11.50am

(1)

Dye
W
No Enz
Xho
Xho/Sal
Xho/Xba
Xho/Xba/Hind
Xho/Hind/Sal
W
Marker
W
Dye

B'49 digests

on 12 noon off 1.0 pm

0.8% H×4 Max

Should be ~14kb

(2)

on 1:28pm off 2:42pm

0.8% H×4 Max

Dye
W
1
2
3
4
5
W
Marker
W
Dye

B'46 Blue Xho

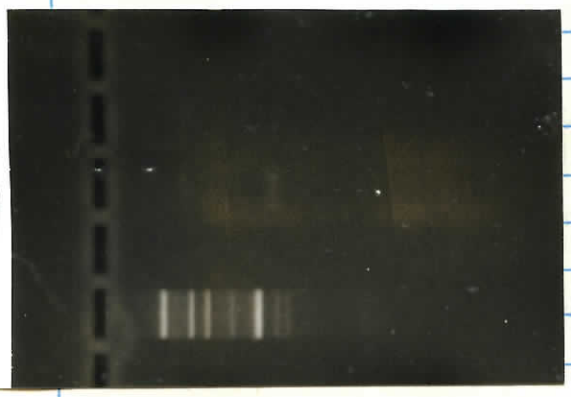
B'46 Blue 2–5 test

12 µl (6×2) 10×R1
107 µl ddw
1.5 µl (6×50) Xho

5×20 µl each plus 1.0 µl minus 2–5 & 2 µl minus 1

B'49 2 1–5 Xho

10:38am → 1:18pm



(1) Conclusion: Some ligated products are visible.

Proceed to prepare more 5' flank

CYN 4:10pm
~~4:40pm~~

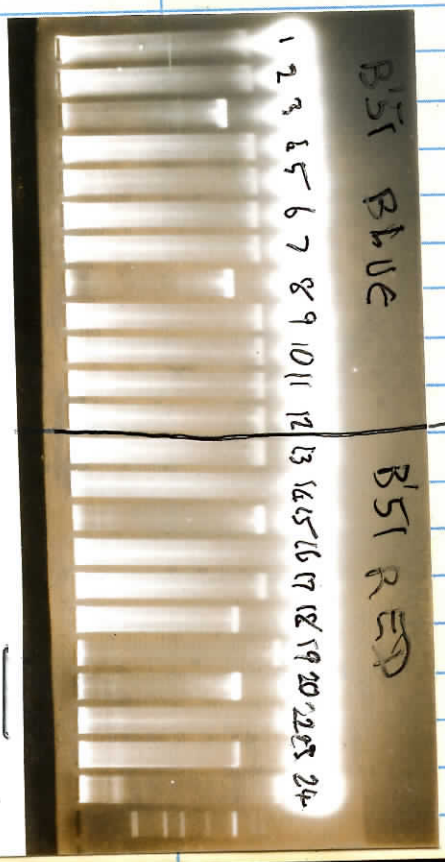
(1)

	Pyr
	W
4 + 12	Blue
	Red
	W
	Marker
	W
	Pyr

10:53am B'51 Minis 1-12 BLUE ← Picked fairly easily
13-24 RED ← Picked only with difficulty.

6pm to stationary

Mini #24 re-seeded from plate at 6:35pm & to shake - it was not growing. #21 is zero on both.



HXG 0.8%

Sun - Feb 13th

3rd (New) ligation for NPR1 Dup

lig^n / lig^n } B'51

Take

✓ 1µl B'45S Not TK Xba
✓ 30µl B'20M Hind Neo Sal

✓ ~35 µl B'43M (Rest) Sal 5' flank Eag

~~on 1.25pm 1.04pm HXG 0.8% Nat~~

32µl → B'51 Blue lig^n
✓ +50µl B'11 148 Blue 3' flank
✓ + 250µl EtOH

32µl → B'51 Red lig^n
✓ +75µl B'11 155 Red 3' flank
✓ + 325µl EtOH

Back into ✓ 10µl 1x p95

+1µl T4 ligase diluted 1 + 4 µl with ✓ 1x p95 ✓

Cold 6.10 pm Sunday → 3:25 pm Monday

Cold 6.10 pm

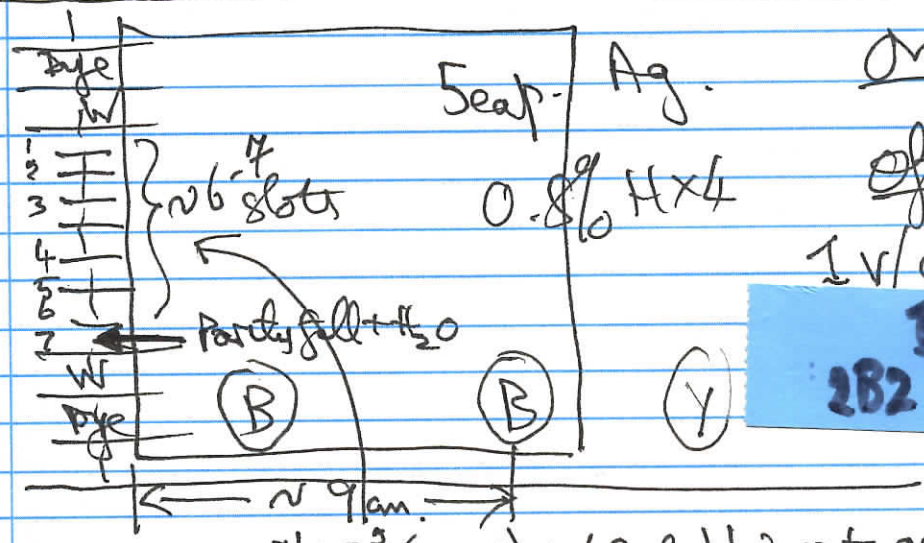
	Blue	Red
Marker	Frag HD H 5x 1/7 150µl	150µl
	Blue lig^n 6µl	6µl Red lig^n
	0°C 3:25pm to 3.55pm	
	90 sec 42°	
	4:13pm Plated 5/16	20/16 5, 20, 40, 40, 40

Pre } B'55 Blue
Post } lig^n
Pre } B'55 Red
Post } lig^n
W Dupl 37°

Conclusion: M infine - proceed.
Total amount available is roughly 10X that visible in gel.

Mon. Feb 14th NM tag

Re-make of Sal [5' Flank] Eag



On 6:28 pm

off ~10.30 am

1 v/cm 100 mA

↑ to 125 mA at 9:15 pm

B'43² 2B2 Eag/Sal

B'43² (30 µl) + 60 µl ddw → to gel
(unfrozen)

Processed to → [B'53 F]

Used Whole → [B'53 M]

Discarded → [B'53 S]

Bye
W
F (Undiluted)
M
S
W
Marker
W
Bye

B'53

On 1 pm

off 1:45 pm

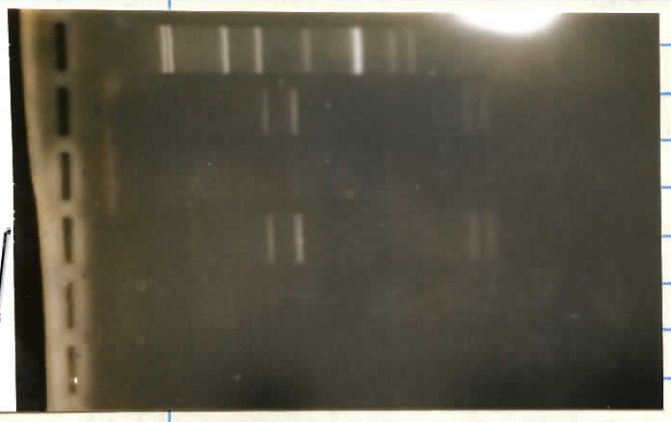
Max 0.8% H×4

	B'54 BLUE	B'54 RED
DH5α 1/7	80 µl	80 µl
B'55 LIG^N DNA	2 µl	2 µl
	0° 5:40	to 6:10
	42°	90 sec
	Plated	NZY 1 Amp
	Rest 20, 5, 20/16, 5/16	
	37° 6:25 pm	
	↓ 10am	

Only ≥1 on B'54 Blue Rest!
0 on Red!

Run gel & decide

See B'51



↑ B'51 gel

Conclusion:
Band production was quite good. Can see the ligated products. Go ahead & repeat transformation with B'55 BLUE & RED ligations — ~3 µl are left 1:48pm Wed.
Meanwhile streaked out the B'54 BLUE #1
Also have another look at B'46 BLUE 1 & possibly pick more of B'51.

See B'64 for B'55 Red

See B'57 — renamed

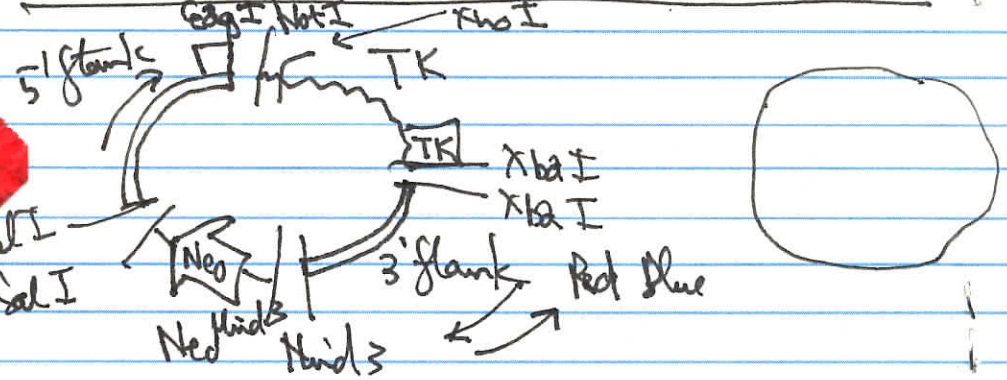
B'55 3 RED
80 µl
Rest ≤ 2 µl
11.50am Tues.
~12.30 Feb 20
✓

B'55 LIGN

2.5
5
10 X 7
12.50 pm Tues
↓
Precipitated Th
↓
B'55 Red 31

Tues Feb 15th

Third attempt at NPR1 DUP construct



B'55 Blue high

B'55 Red high

Take
✓ 8 µl S B'45 Not TK Xba
✓ 60 µl M B20 Hind Neo Sal
✓ Whole M B'53 Sal 5' flank Eag

B'53 M

~130 µl ~130 µl

✓ 100 µl B'11/148 Blue
150 µl B'11/155 Red

Small losses

Extracted 1x HOØ/HOQ
190 µl/230 2 x CHCl3
Transferred to new tube ~250 µl ✓ /280
+ 570 µl. EtOH ~750 µl EtOH

Back into 10 µl pH 5 1X lig^n buffer ✓

✓ 2+13 ✓
B'55 Pre Blue

JL2/18/96
Lot 100035
1/2 µl ✓ + 1/2 µl ✓
Tk ligase ✓ Tk ligase ✓

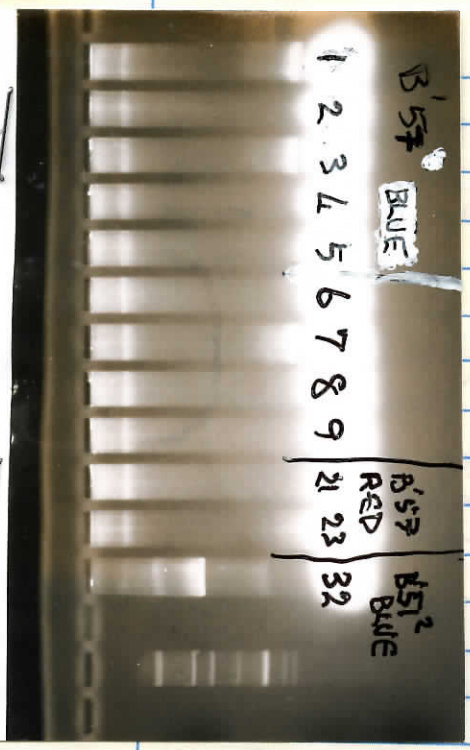
15° 4:37 to 5:07
RT 5:08 to 5:23 ✓
37° 5:23 to 5:38 ✓

✓ 2+13 ✓
Red B'55 Pre

✓ ✓
2+13
B'55 Post Blue

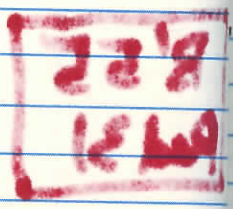
✓ Cold

✓ ✓
2+13
Red B'55 Post



Conclusion: B'51² BLUE #32 (see B'61)

Looks v.v. promising.



(see B'61)

On 8:46am
off 9:46am
0.8% HNE MAX

Dye
W
1
2
3
4
5
6
7
8
9
21
23
32
W
Marker
W
Bye

B'57 BLUE

B'57 RED

B'51² Blue

Wed. Feb 16th

Re-transformation of B'55 B'51 ligation

	B'55² BLUE	B'51² BLUE	B'55² RED	B'51² RED
DH5α 1/7	125µl	125µl	125µl	125µl
DNA LIG Nº	3µl	~3µl (REST)	3µl	3µl (REST)
0°C 2:33pm	3:08pm	3:21 pm	3:14pm pm	3:05pm
	42°	90sec		
Plated	1.5µl	6µl	250µl × 4	
	37° ~ 3:35pm			

(4) (4) (2) (1)

Re-names:

B'54 BLUE

1	≡ B'54 BLUE #1
2	≡ B'51²
3	≡ B'51²
4	≡ B'51²
5	≡ B'51²
6	≡ B'55²
7	≡ B'55²
8	≡ B'55²
9	≡ B'55²

→ B'54 Blue #1

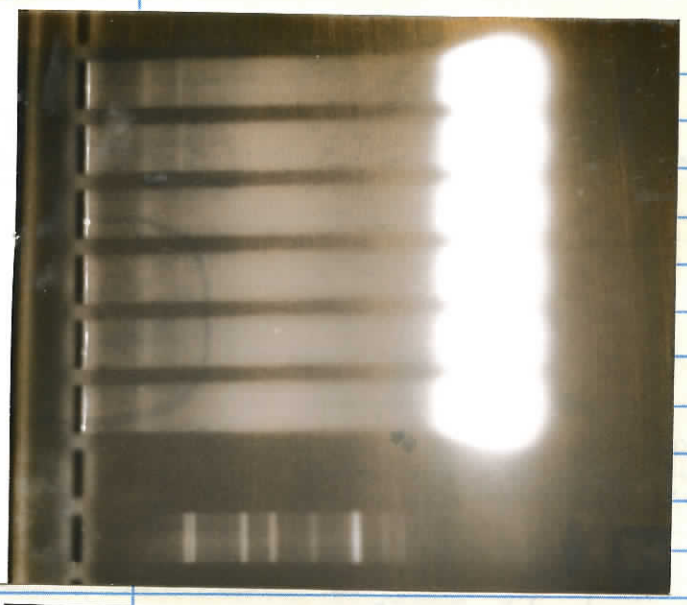
B'57 RED

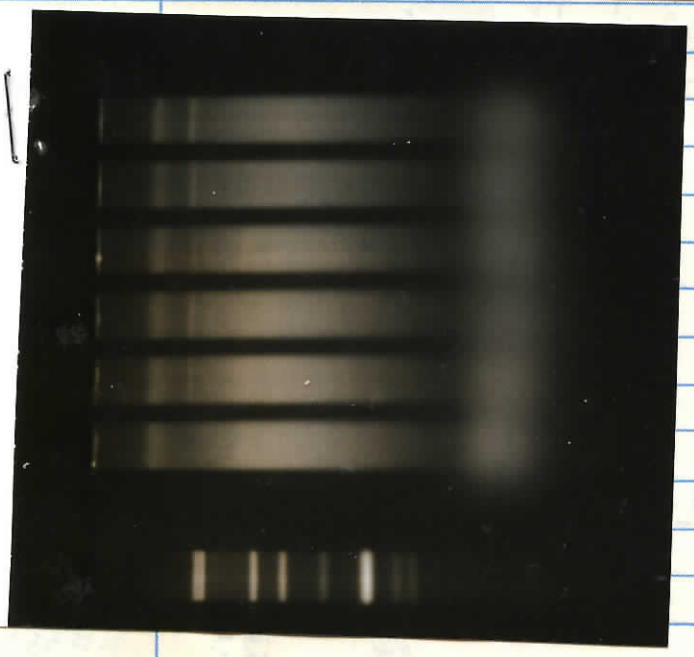
21	≡ B'51²
22	≡ B'55²
23	≡ B'55²

B'57 Minis 1–9, 21–23

37° ~ 10:30am — 7:30am

#22 Red dil not grow





Conclusion:

The old result is not changed appreciably – this is worse!!

[illegible] 1/B'59

Cy 1:52 ~~pm~~ ~~pm~~ 0.86 4x4 MAX

Wed. Feb 16 d

B'46 Blue 1 tests (repeated)

Dye
Water
Water
Xho
X/Sal
X/Xba ✓
X/Xba/Hind
X/Hind/Sal
W
Marker
W
Dye

✓ Take 14µl (7 x 2) 10x R1
✓ 112 ~~104~~ µl dH2O
✓ + * 14 ~~22~~ µl B'46 Blue 1
✓ + 1.5 µl (5v x 6) Xho 1 Lot 26

20 µl ✓

B'59/1 No Enz

Post

4x 20 µl

½ µl ✓ Sal 47 ✓
½ µl Xba 49 ✓
½ µl Xba ½ µl Hind 45 ✓
½ µl Hind ½ µl Sal ✓

All are 20v/µl

B'59/1 Xho
B'59/1 X/Sal
B'59/1 X/Xba
B'59/1 X/Xba/Hind
B'59/1 X/Hind/Sal

37° 11.53am — 1:40pm
Thurs.

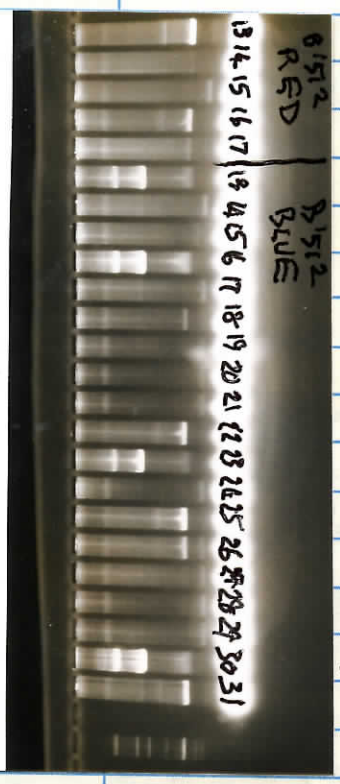
* can't find ~~this~~ mini. One (v. large) colony ∴ into 50 ml 1x AMP NZY → overnight 5:50pm shake

B'46² BLUE 1

✓ 11am ← 9.30 ← 8:50pm
Shake am stationary

Back into 100µl cells etc. looked good → it is single colony

† Changed to 14µl B'46² BLUE 1



Conclusion: ~~At~~ least BLUE looks safe, I work B'51² #13, 16, 23, 30 & 32 all large & the same.

11:30am Processed as usual to 50µl

Fr

B'51² BLUE
13, 16, 23, 30, 32

(contd.)

BLUE
30

on 9.50am
off 11.10am
0.8 HX4
750mA

Thurs. Feb 17th

Rescue of last available colonies B'51

Dye
W
13
14
15
16
17
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
W
Marker
W
Dye

B'51² RED

B'51² BLUE

The Blue & Red plates from B'51 are still available* with very satellited colonies — often with no visible parent colony.

Following were rescued on 2XAMP N2Y plate & 2XAMP cultures

Not all are single colonies

B'51²
13
14
15 (Red)
16
17

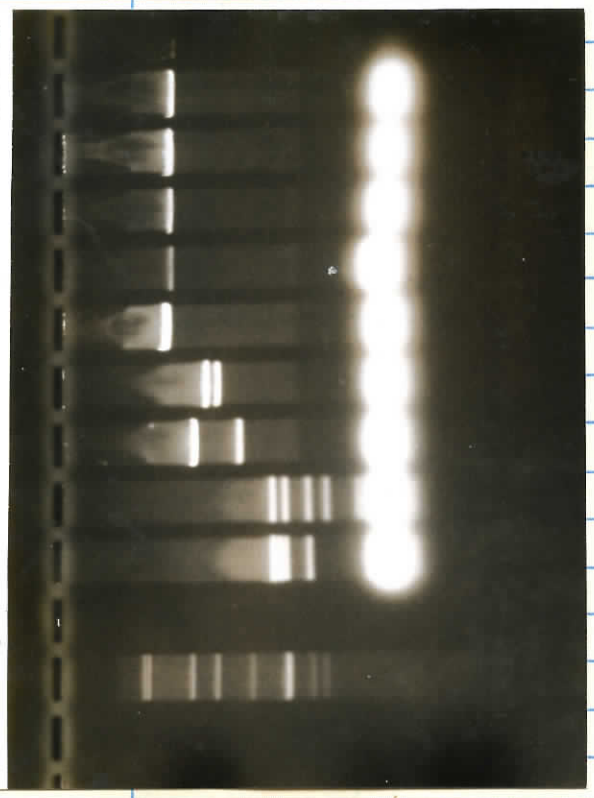
B'51²
13 —
— 32 (Blue)

37° 4:50pm Th.

32 is on B'57 gel

* from the garbage pail!

Processed to KOAc on Fri. Feb 18th estimated 9 am.



Conclusion: These look v. good - but there must be a 1.1 kb band under the RNA in X/Hind/Sal lane. Checking after RNAse confirms OK.

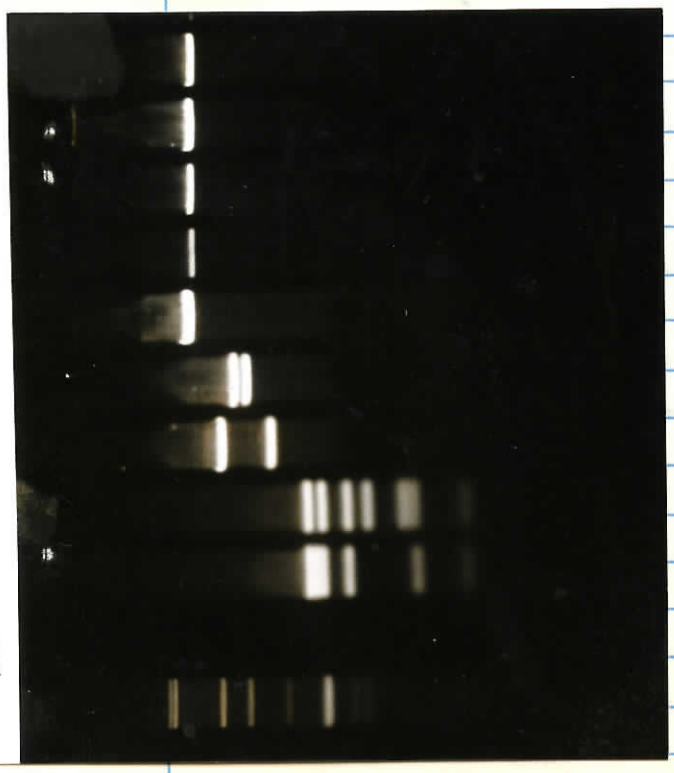
Streaked out ~6pm

B'51² 30

as NPR1 DUP BLUE B'62

Renamed BLUE 31 B'62 see B'73

Orientation needs checking with PCR versus the 3' flank phage. Make RED only if orientation checks incorrectly.



→ Poor tiny colonies. Re-streaked out on fresh NZY 1x AMP plate

→ also B'51² 13, 16, 23, 4, 32, 30 5pm Sat

All colonies are small, but set up 100 ml of NZY 1x Amp one colony from

B'51² 30 10.45am Sun

→ To rt. 11:40am Sun

Fri. Feb 18th

NPR1 DUP Blue tests

Take 24 µl (12×2) 10×R1
213 µl ddw
(12×5) 3 µl Xho 1 Lot 26

20 µl → 13 Xho
20 µl → 16 Xho
20 µl → 23 Xho
20 µl → 32 Xho
Rest → 8 µl 30

B5 numbers + 1 µl 13 Xho
+ 1 µl 16 Xho
+ 1 µl 23 Xho
+ 1 µl 32 Xho

~~5:00 pm~~ 37° 12:49 pm

0.8% HX4 MAX

4× 20 µl

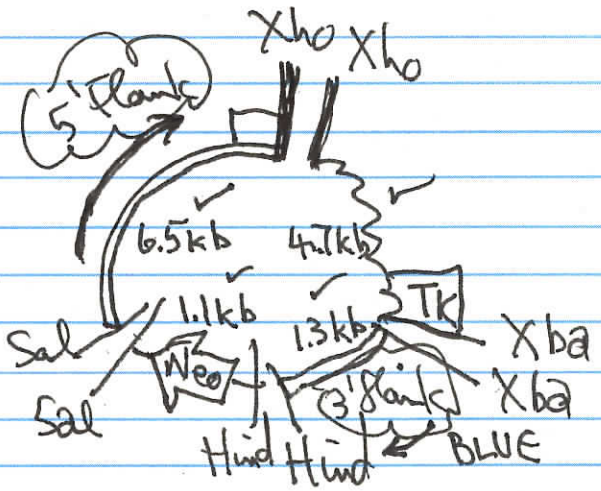
Rest → 30 Xho
+ ½ µl Sal 47 → 30 X/Sal
+ ½ µl Xba → 30 X/Xba
+ ½ µl Xba, ½ µl Hind 45 → 30 X/Xba/Hind
+ ½ µl Hind, + ½ µl Sal 47 → 30 X/Hind/Sal

Dye
W
13
16
23
32
30
30 X/Sal
30 X/Xba
30 X/Xba/Hind
30 X/Hind/Sal
W
marker
W
Dye

B'63 Xho digests undil'd.

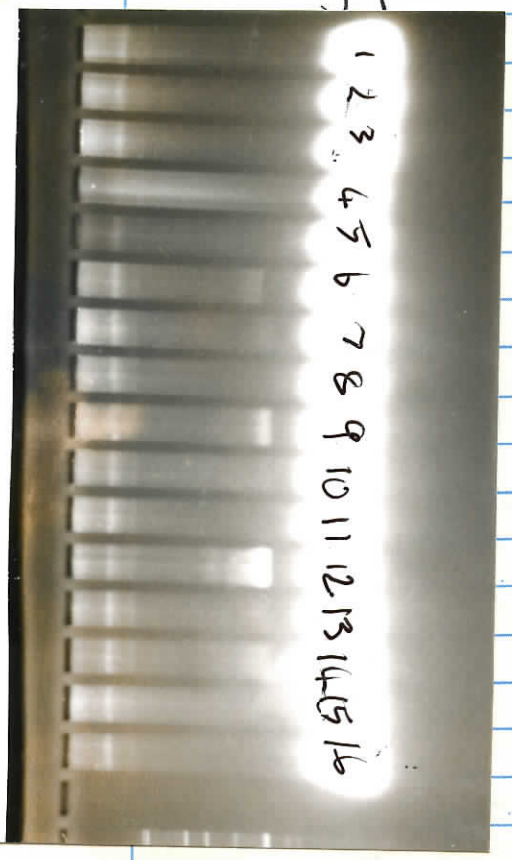
37° 12:49 pm to 3:30 pm

B'63 digests undil'd



Processed to KOAc — all had ~ normal (low) brite
Looking pellets.

(1)

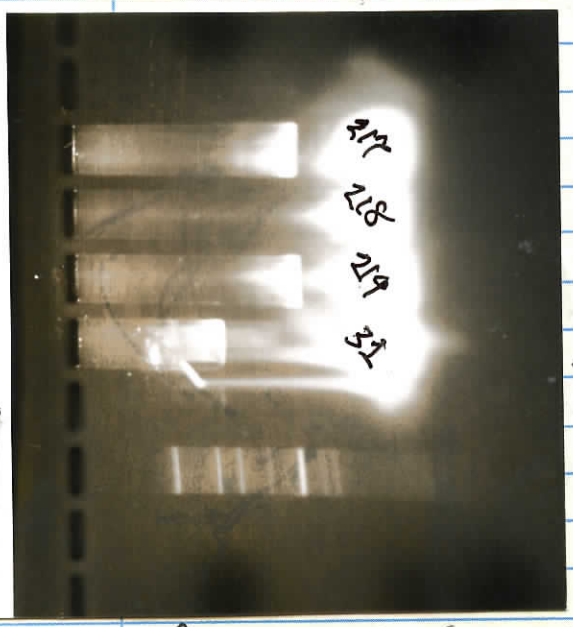


Conclusion: None are correct!
Try rest of B'55 (as B'55³)
and 2 µl more of B'65
(as B'65²)

Did so. 3 colonies B'65²
from B'65² [217, 218, 219] RED
1 colony from B'55³
[B'55³ 31] RED

To 37° as minis ~3:20pm
2 X Amp
Tues. Feb 23rd
2 streaked out
on NZY 2 X Amp
~9:30am Th bright
growth but all OK

(2)



Red 31

Dye	On 10:20 Off 10:50
W	
217	B'65²
218	
219	
31	B'55³
W	
Marker	
W	
Dye	

Conclusion: looked as if B'55³ #31 is good, See B'69.

Sat Feb 19th

Repeat of NPR1 DUP RED ligⁿ (B'51)

Dry 10:02am
Off 11:04am
0.56 H x 4 MAX

B'65 running

25 bpl
✓ Take 1 µl B'45 S Not TK Xba
✓ 30 µl B'20 M Hind Neo Sal
✓ Whole (~150 µl) B'43 F
✓ 75 µl B'11 155 Red 3' flank

B'43 F

+ 768 µl EtOH

Back into 10 µl 1x p95 ligⁿ buffer
+ 1 µl [1 µl + 4 µl p95 T4 ligase] JL tube

Possibly a little more

Cold 6:20 pm

B'65 RED LIGⁿ

10:50 am Sun

B'65²		B'65	
✓ 80 µl	DH5α 1/7	~200 µl	✓
✓ 2 µl	B'65 Red ligⁿ	✓ 4 µl	
	0° 10:53 am		11.24 am
	90 sec	42°	
	Plate of N2Y	1 X AMP	
		Total	~12

B'65 2

B'65 Rest

Tues 11.56 am
Feb 22 ~12:30

2.5, 5, 10 x 7
12:50 pm Tues

2, 4, 8, 16, 20 20 20 20 20 20 Rest

37° 11.40 am Sun

12 colonies (all of variety but #4 & #15) to mini

9 am Mon

9.24 am Mon → 5 pm 5 µl to new replica plate → 9 am Tues

Wed Feb 23rd

Using up last of B'65 Lig^n

DH5α 1/7	240 µl	DH5α 125 µl 10.3 Φ25
Lig^n	B'65 Red Lig^n ~4 µl	3 µl B'65 Red Lig^n
0°	4:04 pm 4:45	434 pm
42°		90 sec
Plated	2.5, 5, 10, 20 ----- (25)	1.25, 2.5, 5, 10, 20 ---- (13)

37° ~ 5 pm

~ 9.30 am

Stored in cold — pending analysis of B'55 Red 31

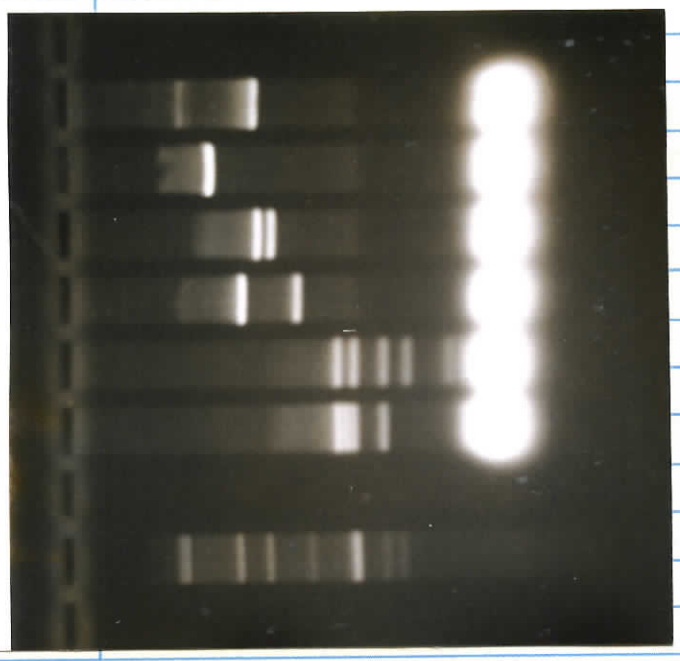
Thur. Feb 24th Discarded

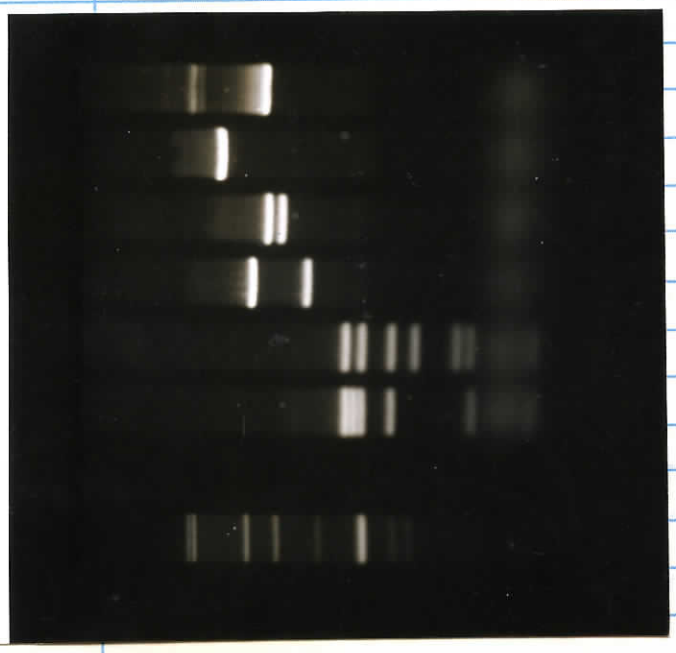
DH5 1/7

DH5 10.3

B'65 RED LIG

Streaked out for Sylvia on suitable plate Minimal medium





Conclusion:

Self-evident!

On 3:59 pm
Off 5:10 pm
0.8%

Undiluted

Pyc
W
Uncut
X/Xho
X/Sal
X/Xba
X/Xba
X/Xba
W
Marker
W
Pyc

Thurs. Feb. 24th

Red

NPR1 DUP Red B'55

B'69 Red 31 digest

Take 14 µl (7 x 2) 10 x R1

117.5 µl dd w

7 µl B'55 Red 31

(6x5u) + 1.5 µl Xho I Lot 26

Hind Sal

B'69 31

20 µl

Rest

½ µl Sal 47 ½ µl Xba 29 ½ µl Xba Hind 45 ½ µl Hind ½ µl Sal 20

20 µl

B'69 Uncut

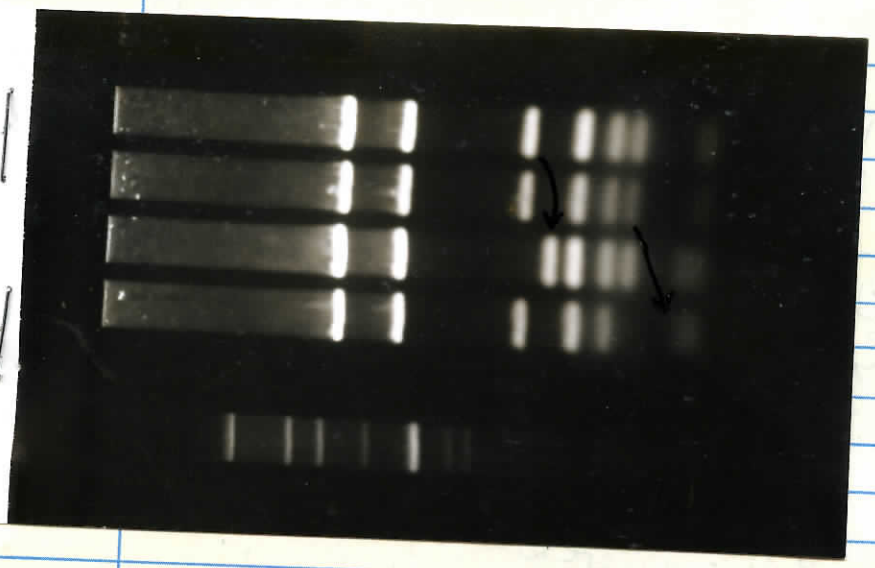
B69 Xho | X/Sal | X/Xba | X/Xba/Hind | X/Hind/Sal.

37° 12.45pm → 3:45pm

Bye
W
Uncut
Xho
X/Sal
X/Xba
X/Xba/Hind
X/Hind/Sal
W
Marker
W
Bye

3:59pm
5.10pm
0.8% H Xb

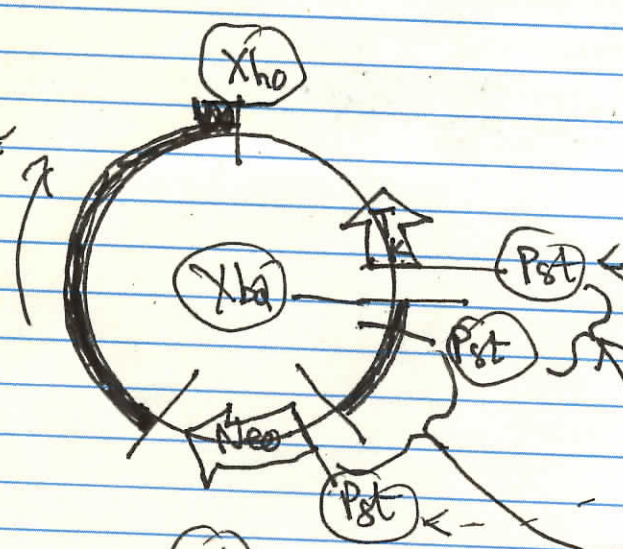
MBK



Conclusion:
The Pst patterns are identical (see * below) but the Pst/Xba differ & allow orientation to be determined, but beware of PCR using any promoter primers

SHS 154
B'55³ Red
31
1.275 µg/µl

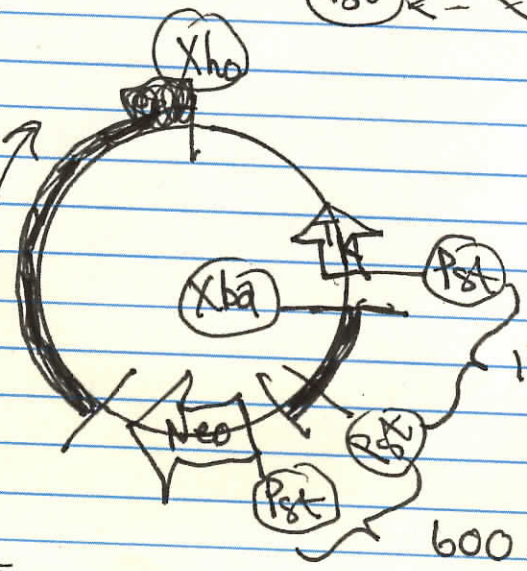
RED 31



* Same site because both use pMC1 promoter & enhancer

600 → 2×300 with Xba
1300 does not change

BLUE 30



1300 → 300 + 1000 with Xba

600 does not change

SHS 148/158
Blue DupNPR1
B'62 0.775 µg/µl

Rename B'62 Blue 30 see B 13

on 11:42
off 12:47
0.86 HX4 max

RED 31 | BLUE 30

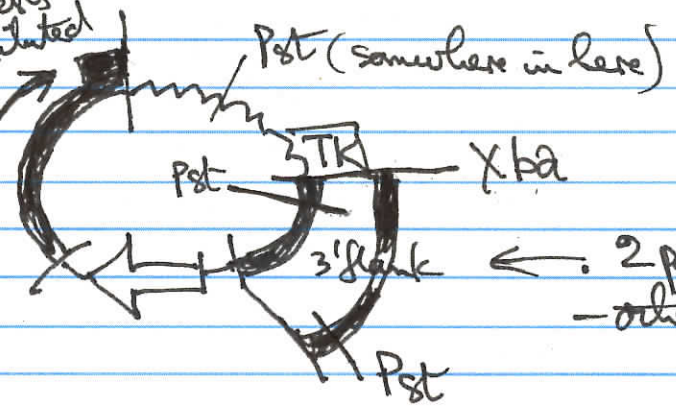
Fri. Feb 25th

Cross-checks of Red 31 v. Blue 30

Bye
W
Pst Blue
Pst Red
Pst/Xba Blue
Pst/Xba Red
W
Markers
W
Bye

B'71 digests undiluted

5' blank



← 2 poss. orientations
– other Pst sites unknown

∴ take ✓ 10 µl 10x R1 salts
✓ 80 µl ddw
+21.5 µl (50u) Pst I lot 35

✓ 40 µl → +2 µl Blue B'512 30 Mum
40 µl → +2 µl Red B'55 31

✓ 20 µl → ✓ + ½ µl (10u) Xba I lot 29
20 µl → ✓ + ½ µl (10u) Xba I lot 29

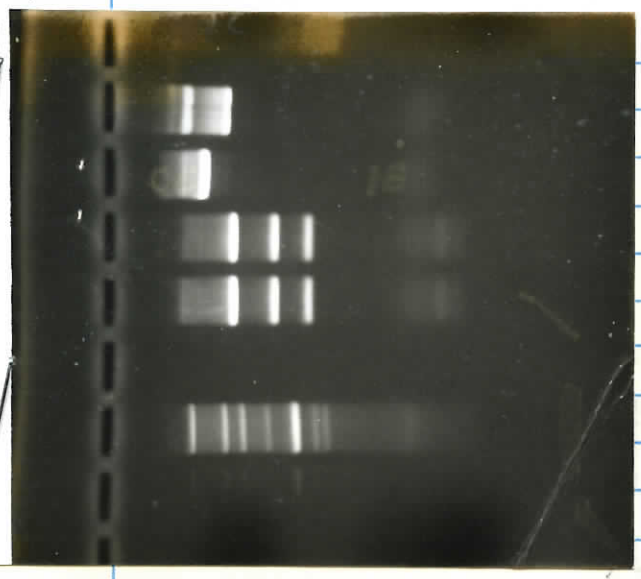
Blue PX	Blue P	Red PX	Red P

37° 9:54 am
11.29 am

See PMO 3-127.

The desired orientation for duplication is RED 31

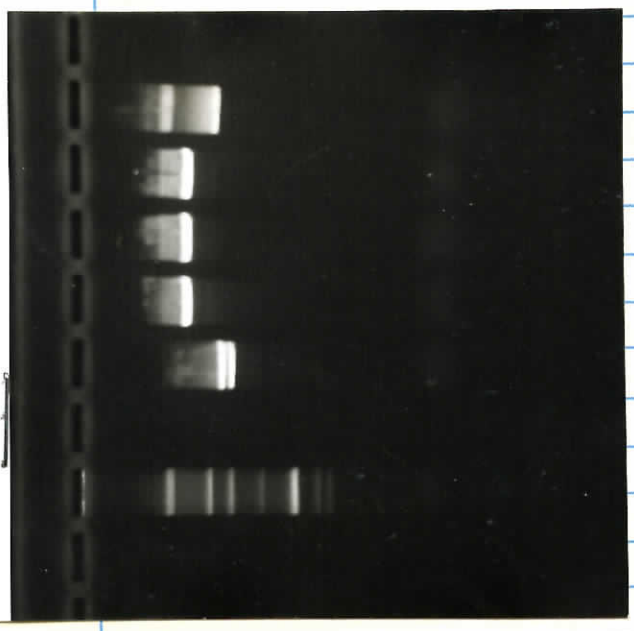
(1)



(1)

Conclusion: B'62 BLUE3D has Xho as expected, but too many Eag sites, including one close to Xho. Try Not I (~ 1 in 4 chance assuming none elsewhere)

(2)



(2)

Conclusion: B'62 BLUE3D has the Sal site expected & generates the correct Sal/Xho fragments. But there is no Not I site. Can proceed by changing Xho to Xho Not Xho again & reinserting {Sal Eag Reversed} into Sal Not.

BLUE 30

Sat. Feb 26th

Conversion of DUP to DEL(ete)

Can excise ~~the~~ Sal [5' Blank] Eag ~~frag~~t

~~from the~~ incorrect Red or Blue & invert it by site switching — or many ~~other~~ possibilities. Check if Eag site is unique (& ~~Sal~~ present).

Sylvia prep of B'62 BLUE NPR1 DUP — now ~~pre-named~~ B'62 Blue 30

~~Off 12:28 pm~~ 11:15 am

0.8 µg HX4 MAX

① Bye, W, Uncut, X, XE, E, W, Marker, W, Bye

Take ✓ 8 µl (4×2 µl) 10× R1 salts
✓ 71 µl ddW
✓ (0.8 µg) 1 µl B'62 BLUE 30

BLUE 31 ligands undiluted

Rest → Uncut B30

20 µl → ½ µl Xho I Lot 26 → X B30

20 µl → ½ µl Xho + ½ µl Eag I Lot 22 → XE B30

20 µl → ½ µl Eag I → E B30

37° 10:43 am → 11:45 am

~~Off 5:20 pm~~ ~~Off 6:06 pm~~ 0.8 µg HX4 MAX

② Bye, W, Not (N), Xho (X), Not + Xho (NX), Sal + Not (NS), Sal + Xho (XS), W, M, W, Bye

Take ✓ 10 µl (5×2 µl) 10× R1 salts
✓ 89 µl ddW
✓ 1.2 µl B'62 BLUE 30

Check on Not I site

50 µl → ½ µl Not I 46 → NX B30

Rest → ½ µl Xho 26 → X B30

Rest → 20 µl → ½ µl Sal I → N B30

20 µl → ½ µl Sal I → NS B30

20 µl → ½ µl Sal I 26 → XS B30

3:40 pm → 5:10 pm

Forward

Ace Summary

ACE 1 (~~rev~~ +): 6/11/93

DNA: SHR25 w110 #7 xhol
2 Electroporations 3ul DNA and 30ul DNA
Pick 40 colonies and froze 20 colonies
2 vials per colony.

Positive control for ACE-DUP

ACE 2 (rev +): 6/25/93

DNA: SHR35 w157 "8"
2 Electroporations 3ul DNA and 30ul DNA
Pick 20 colonies. Froze 2 vials (or more) from 16 colonies.

This is the correct one to use.

Positive control for ACE-DUP with Neo Reversed

ACEDUP-1: 7/10/93

DNA: SHR59 Rev6, A'19 Rev 10ug SH
1 Electroporation with 10ug DNA
Pick 180 colonies, all negative.

ACE-DUP with Neo Reversed

ACEDUP-2: 7/14/93

DNA: SHR59 Rev6, A'19 Rev 10ug SH
1 Electroporation with 10ug DNA
Pick 120 colonies, 8 positive lines.

ACE-DUP with Neo Reversed

Cell line	# vials frozen	chromosome % at 40	injection date(s)	result
7C	12	0%, 70%		
15A	8			
6D	10	80%		
2B ← S	8	62%, 100%	8/23/93	2 chimeras - weak
			8/25/93	no chimeras
			9/8/93	Not preg.
			9/9/93	No chimeras
3B ← S	11	66%, 80%	10/6/93	Not preg
3D ← S	7			
5A	12	80%		
4B	8			

Only ones from here

4 ... but show ... meaning ... then OK

Thaw 3D for chromosomes

Thaw 5A 6D for Southern

Part M is really
AGT → ANG I
not total
removal

which must
equal production

Tues. March 29th

Some numbers on renin / angiotensin

Assumptions:

(i) 1, 2, 3 & 4 gene copy animals are to a first approximation normotensive.
(ii) all corrections are via renin conc^n changes.
(iii) angiotensin II production is constant (corollary of assumption (i) or (ii))
(iv) angiotensin II production changes linearly with angiotensinogen and/or renin conc^{ns}
(v) the duplication chromosome is not twice normal.

Normal $2p - u = 100$ (p = production from one normal chromosome. u = utilization of AGT)

1/0 $p - u = 35$

$\therefore p = 65$ and $u = 30$

2/1 $x.2p + p - u = 124$ (x = fractional efficiency of an AGT gene in duplicated case)

$\therefore x = 0.685$

Predicted 2/2 $2.x.2p - u = 148$

Predicted 2/0 $x.2p - u = 59$

This looks good → $[100]$ (normal AGT level) $\times R_2$ (normal renin level) $= 100\%$ ATI production $\quad R_2 = 1$ (assumed)

$\therefore$ Predicted 1/0 $R_1 = 2.86$

Predicted 2/1 $R_3 = 0.81$

Predicted 2/2 $R_4 = 0.68$

Predicted 2/0 $R_{2/0} = 1.69$

Predicted 0/0 $R_0 \equiv$ off scale

If normotensive

Wed. April 6th

Another try!

Let clearance be prop.^{l} to [AGT]
Let util.^{n} - - - - - - - - [AGT] → constant for 1, 2, 3
Let 2AGT drunk/some be $2x$ * normal

1/1 $\quad 2p = u_{100} + c_{100}$

1/0 $\quad p = 0.35u_{100} + 0.35c_{100} = 0.35(u_{100} + c_{100})$

2/1 $\quad 2px + p = 1.24u_{100} + 1.24c_{100}$

2) are inconsistent.

Try u constant (for normolentivenets) with clearance prop.^{l} to [AGT]

1/1 $\quad 2p = u_{100} + c_{100}$

1/0 $\quad p = u_{100} + 0.35c_{100}$

1) − 2) $\quad p = 0.65c_{100}$

and $u_{100} = (0.65 - 0.35) = 0.3c_{100}$

or $c_{100} = 1.54p$

cf. B'79

2/1 $\quad 2px + p = u_{100} + 1.24c_{100}$

3) − 2) $\quad 2px = 0.89c_{100} = 0.89 \times 1.54p$

$\therefore x = \frac{0.89 \times 1.54}{2} = 0.6853$ c.f. B'77

Next:

2/0 $\quad 2px = u_{100} + q \cdot c_{100}$ ← AGT level

$\therefore 0.89c_{100} = 0.3c_{100} + q \cdot c_{100}$

and $q = 0.89 - 0.3 = 0.59$

AGT 2/0 59%

Remin levels are 1/1 = 1.0
1/0 = 2.86
2/1 = 0.81
2/0 = 1.69

Predictions

See also Bio5

Sat. April 18th

Further numbers

Assume production is just linear with gene dosage;
Assume 1, 2, 3 copy animals are normotensive & "therefore" make same amounts of ATI
Assume non-specific clearance is proportional to [AGT].

1) 1/1 $2p$ − Clearance at 100% plasma level C_{100} = Utilization to make ATI $= u$

2) 2/1 $3p - 1.24\,C_{100} = u$

3) 1/0 $p - 0.35\,C_{100} = u$

From 1) − 3) $p = 0.65\,C_{100}$ or $C_{100} = 1.54\,p$

But from 2) − 1) $p = 0.24\,C_{100}$

This is not possible.

All of which makes the clearance (??) receptor of ANF more interesting.

2/2 5) $4px = u_{100} + r\,C_{100}$

But $p = 0.65\,C_{100} \rightarrow u_{100} = 0.3\,C_{100} \rightarrow x = 0.6853$

$\therefore r = 4 \times 0.65 \times 0.6853 - 0.3 =$ 148% AGT 2/2

With renin 2/2

2/2 = 0.68

600153
4/26/94

STRATAGENE

Certificate of Analysis

PRODUCT: **Cloned *Pfu* DNA Polymerase**

CATALOG #: 600153 600154 600159
(40 µl)

QUANTITY: 100 U 500 U 1000 U

CONCENTRATION: 2500 U/ml 2.5 u/µl

SPECIFIC ACTIVITY: 75,000 U/mg

LOT #: **163006F**

CERTIFIED BY: Mila Angert M. Angert

DATE: April 11 ,1994

QUALITY CONTROLLED BY: Jeff Amberg Jeff Amberg

DATE: April 14, 1994

DESCRIPTION: Cloned *Pfu* DNA polymerase is isolated from the hyperthermophilic marine archaebacterium, *Pyrococcus furiosus*. The multifunctional thermostable enzyme possesses both 5′ to 3′ DNA polymerase and 3′ to 5′ exonuclease activities. The 3′ to 5′ proofreading activity associated with cloned *Pfu* DNA polymerase results in a 12-fold increase in fidelity of DNA synthesis over *Taq* DNA polymerase. Cloned *Pfu* DNA polymerase has a temperature optimum between 72–78°C, and remains greater than 95% active following one hour incubation at 95°C.

REACTION BUFFERS: **10× Cloned *Pfu* Polymerase Reaction Buffer***
200 mM Tris-HCl (pH 8.75)
100 mM KCl
100 mM $(NH_4)_2SO_4$
20 mM $MgSO_4$
1% Triton® X-100
1 mg/ml BSA

REACTION CONDITIONS: **Note: All reagents, including cloned *Pfu* DNA polymerase, should be mixed immediately before use.**

DNA synthesis is performed in 100 µl of a mixture containing 20–200 µM dNTPs, 0.3–1 µM primers, 0.1–250 ng template DNA, 10 µl 10× polymerase buffer and 2.5 U *Pfu* DNA polymerase. Mix the reaction gently, centrifuge briefly, then overlay with light mineral oil. Initially, denature the reaction by incubating at 95°C for five minutes; then cool to 40–68°C for 5 minutes to allow primers to anneal to the template DNA; then incubate at 72–78°C to initiate primer extension.

***Formerly sold as Buffer #3.**

11011 North Torrey Pines Road, La Jolla, CA 92037
Ordering / Technical Services: (800) 424-5444 or (619) 535-5400

Tag.

Tues. April 26th

Simple test of Lehrman Barnes' principle with ATR1B amplification

Conditions for 1.2 kb PCR have been borderline
∴ test ± editing Taq (Pfu) – using $\frac{1}{10}$th vol, (intended $\frac{1}{20}$)

Usual formula (see A'121):

				Barnes
	d.d.w.	$n \times 2.85$ µl		
pH 8.8	20 × PCR A'121	$n \times 1.25$ µl	≡ 67 mM	pH 8.6 20 mM + extra Tris to pH 9.1
	4 dNTP's 25 mM	$n \times 0.1$ µl	≡ 250 µM	250 µM
	DMSO A'121	$n \times 2.5$ µl	≡ 10%	None
	Kwiatkowski 139 3' Neo primer	$n \times 0.3$ µl		
	A'102 ATR1B primer	$n \times 1$ µl		
	Standard Taq A'121 + B'81	$n \times 2$ µl		
		$n \times 10$ µl		
			16 mM	16 mM $(NH_4)_2SO_4$
		$+ n \times 15$ µl DNA sample	6.7 mM Mg	3.5 mM Mg

This is 1/10 of Taq by volume

Rest 60 µl + 1.4 µl Pfu B'81

Today n = 12

34.2 ✓
15 ✓
1.2 ✓
30 ✓
3.6 ✓
12 ✓
24 ✓
120 µl

5 × 10 µl ✓✓✓✓ 5 × 10 µl ✓✓✓✓

	d.d.w.		
1	10 µl	5 µl 6E	✓
2	13 µl	2 µl 12D	✓
3	10	5 7B	✓
4	10	5 16C	
5	5	10 16	✓
6	10 µl	5 µl 6E	✓
7	13 µl	2 µl 12D	✓
8	10	5 7B	✓
9	10	5 16C	
10	5	10 16	✓

6E, 2D, 7B, 16C, 16, 6E+, 2D+, 7B+, 16C+, 16+

PCR mix (modified from the recommendations of New England Biolabs) is 67mM Tris-HCl (pH 8.8), 6.7mM $MgCl_2$, 16.6mM $(NH_4)_2SO_4$, 10mM 2-mercapto-ethanol, 6.7µM Na_2EDTA, 10% dimethylsulfoxide, 330 µM of each dNTP, 3.0 µg of each primer, 1 unit Taq polymerase per 50 µl.

∴ modify by ± $n \times 0.1$ µl of Pfu

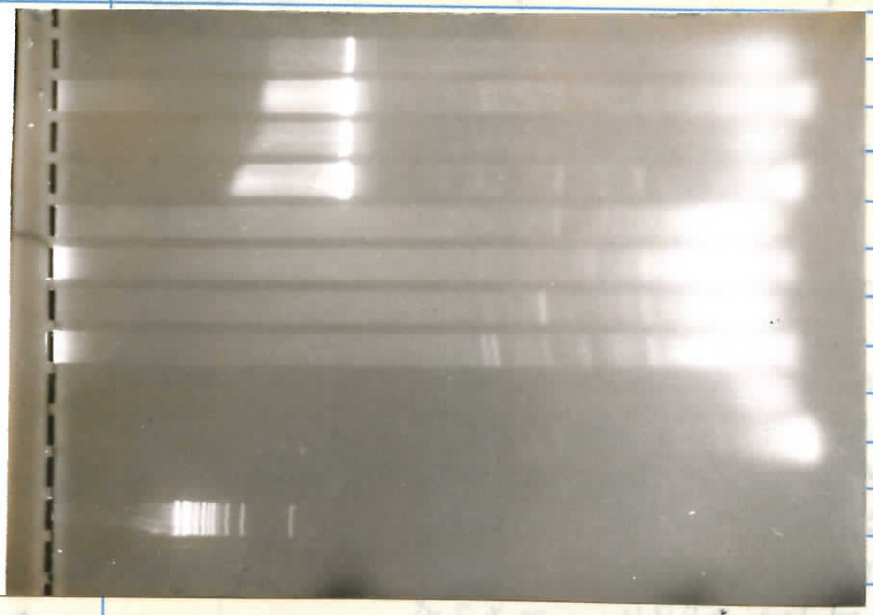
Positive controls are { Pos. Control A109 6E, try 5 µl } and { Pos. Control A109 12D, use 2 µl }

Finished → Test { A111-4 7B, A111-4 16C, Use 15 µl } – these are real clones

	Temp	Time
Bath	95°	92°	Total 1 min
39°	56°	Total 7 sec
60°	60°	10 min

35 cycles

A111-4 16 } – this is the +ve pool
Use 15 µl }
On 5.15 pm 1st cycle not v. good
are temps.



On 10:50am 10V/cm 300mA 2% Ag HX1
Off 11:38am

Dye
Water
1 − } 5µl A'109 (6F)
6 +
2 − } 2µl A'109 (12D)
7 +
3 − } ~5µl A'111-4 (7B)
8 +
4 − } 5µl A'111-4 (16C)
9 +
5 − } 10µl A'111-4 (Pool)
10 +
W
Ag Marker
W
Dye

Conclusion: The Pfu definitely can be beneficial. However 7B & 16C show the unexplained failure mode. Simon thinks that too many cycles can cause this. Wayne says it occurs more frequently when larger amounts of enzyme are being used. ∴ test both variables ∴ less enzyme & fewer cycles. Don't have much sample left ∴ develop technique with ES cell DNA isolated by Dennis from our current injection cell lines.

On 11.05 am

30 — 35 — 1 + Pfu etc. } 1x Taq

30 — 35 — $\frac{1}{5}$ x Taq

Trouble initiating →

	1	−
	4	+
	2	−
	5	+
	3	−
	6	+
	1'	−
	4'	+
	2'	−
	5'	+
	3'	−
	6'	+
	7	−
	10	+
	8	−
	11	+
	9	−
	12	+
	7'	−
	10'	+
	8'	−
	11'	+
	9'	−
	12'	+
	W	
	Marker	
	W	
	Dye	
	Dye	

Standard Taq + Pfu

Taq/5 + Pfu

Conclusion: The level of Taq that I have been using is close to 1.5x more than nec.
35 cycles is better (but not greatly) than 30 cycles.
The Pfu was not effective at this level — it seems to ↑ shorter fragment.
looks as if the DNA is poor. Try ϕOH/HOQ, CIAA & repeat with 0.4μl Taq/25μl and with 0.04μl Pfu/25μl, 35 cycles.
Also vary amount of DNA. Use only ½ μl of control
N'109 12 D

6V/cm 600mA
off 11.45am

2% HX

Wed. April 27th

More Pfu tests with 1µl Taq / 25µl (50% B'81) or 0.2µl Taq / 25µl (10% B'81) and 1/20th vol Pfu (50% B'81)

Set up for 30 samples:—

n= 30

ddw	536 µl
20xPCR	37.5 µl
4dNTP's	3 µl
DMSO	7.5 µl
Kim's W139 3' Neo primer	9 µl
A'102 ATR1B primer	30 µl
30 x 23 µl = 690 µl	

Check that in future — it is OK by the book. C'th probably use 15µl

12D
16C
7B
30 cycles

12D
16C
7B
35 cycles

12D
16C 30 cycles
7B

325 µl — 325 µl

+14 µl Taq 1 — + 2.8 µl Taq 1

Rest for del^n of Pfu

0.5µl B'81 Pfu + 9.5µl Pfu/10

165 µl — 165 µl — 165 µl — 165 µl

(48 µl) — Positive Control — Diluted sample 1:10 in water — +3.6 µl Pfu/10 — (48 µl) — 0.72 µl Pfu/10 — (48 µl) — (48 µl)

12D 35 cycles

+1µl Taq — A'109 12D — A'111-4' 16C — A'116-4 7B — +2µl — A'109 12D — 16C — 7B — +2µl — A'109 12D — 16C — 7B — +2µl A'109 12D — 16C — 7B

1 1' 2 2' 3 3' 4 4' 5 5' 6 6' 7 7' 8 8' 9 9' 10 10' 11 11' 12 12'

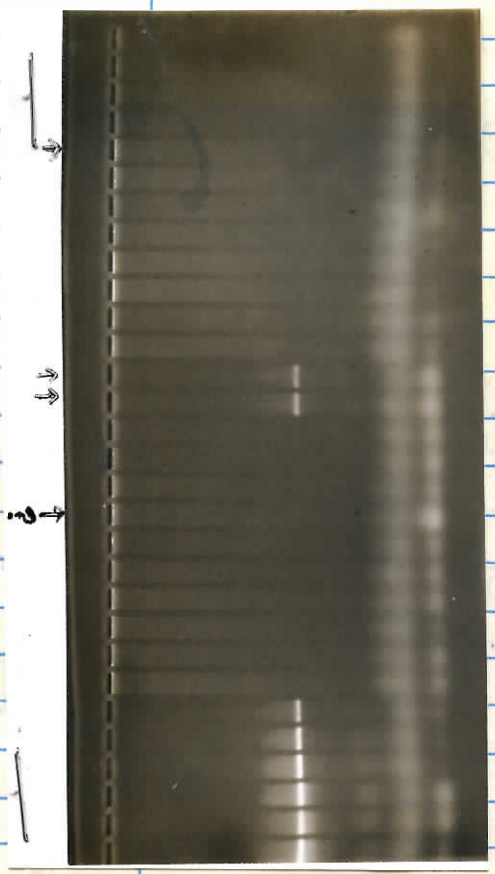
12D Taq 1 — 16C 7B — 12 Taq Pfu — 16C 7B — 12D Taq 5 — 16C 7B — 12D Taq 5 Pfu — 16C 7B

Heated at 60°
1st cycle 93°
later 92°
Good start

1st cycle began ~ 3:42pm
Take out the non-prime set at 28-30 cycles
Final Total ~ 9pm

Temp	Time
92°	1min
61°	1 sec.
60°	10 min

9.10pm Removed the non-prime set after 30 cycles — left 1 min or less
Primeset went full 35 cycles



M 9:35am
of Yellow try to wick

No Pfu	1 μl DNA (~50ng)	1
		2
		3
		4
	16C	5
	~100ng	6
		7
		8
		9
	~150ng	10
		11
		12
+ Pfu	16C, 16CP	1'
	~50ng	2'
		3'
		4'
		5'
	~100ng 16CP	6'
		7'
		8'
		9'
	~150ng	10'
		11'
		12'
Control	1 μl	13
	2 μl	14
	3 μl	15
Control + Pfu	1 μl	13'
	2 μl	14'
	3 μl	15'

Conclusion: Clearly with control the +Pfu is better but linearity with DNA conc. is lost.

PT 111-4 16C is positive in two tests of three

Clear that the problem is likely to be licked by ↓ DNA, & the Pfu helps. Blend no clear help.

Try again with 5 ng and 10 ng of DNA ± Pfu

Also try with NaOH denat.

~1.2% ~ HX2
600mA

Thurs April 28th

More PCR tests

A111-4 16C 1.6 µg/µl and A111-4 B 1.06 µg/µl

Take 200µl of each. 1x HOØ/HOQ
2xCIAA

B'87 16C A111-4 Ø — Red

B'87 B A111-4 Ø — Red

Make :—

16C 16C P(henol) at 0.05 µg/µl etc

i.e. 95µl T/E buffer
+3.1µl 16C or 3.1µl 16CP + 4.7µl 4B or 4BP

B'87 16C 50ng/µl — Blue

B'87 16CP 50ng/µl

B'87 B 50ng/µl — Blue

B'87 BP 50ng/µl

7B

Set up for 30 samples

3:38pm		Start
Boil 25	92.5°	1 min
40	~61°	7sec
60	60°	10min

Good load at 60°

ddw 536 µl
20xPCR 37.5 µl
4xNTPs 3 µl
DMSO 75 µl
W1393 Neoprimer 9 µl
A102 ATRI B primer 30 µl

30×23 = 690 µl

+ 12 µl Taq

0.4u | 25µl

Rest [illegible] µl

345 µl + 0.6 µl Pfu B'81

29 µl −Pfu

29 µl +Pfu

	1	2	3	4	5	6	7	8		1'	2'	3'	4'	5'	6'	7'	8'
1µl	16C	16CP	7B	7BP	16C	16CP	7B	7BP	2µl	ditto							
	9	10	11	12	13	14	15			9'	10'	11'	12'	13'			
3µl	16C	16CP	7B	7BP	½µl	1µl	1.5µl					13'	14'	15'			

Conclusion: Went backwards!
Return to 1 µl of Taq

Run 12:07 pm

	Dye W	
2 µl	1	C/2
	2	C/4
	3	C/8
-Pfu	4	B/2
	5	B/4
	6	B/8
	1'	C/2
	2'	C/4
+Pfu	3'	C/8
	4'	B/2
	5'	B/4
	6'	B/8
W		
-Pfu	30	Alkali denat B
	31	C
+Pfu	30'	B
	31'	C
	W	
	Marker	

Note reverse order of above

For alkali denat start should really hold 60° for 10 min.

5:20 pm
Good 60° followed by 92° start

	Max. temp
1:00	93°
7 sec	~61°
10:00	60.1°

35 cycles

	B'87	Dye
	16 C 50 ng/µl	7 B 50 ng/µl
✓✓	10 µl	10 µl
✓✓	+ 10 µl TE	+ 10 µl TE
B'88	C/2	B/2
✓✓	5 µl	5 µl
✓✓	+ 15 µl TE	+ 15 µl TE
B'88	C/4	B/4
✓✓	2.5 µl	2.5 µl
✓✓	+ 17.5 µl TE	+ 17.5 µl TE
B'88	C/8	C/8

1.4% H × 1 ~ 8V/cm

Fri April 29th

PCR Contd.

May need revision to keep salt conc^n lower — this will give ~ M final

C/2 ≡ 50 ng

1:8 dilutions B

Test standard method ± Pfu with DNA levels decreased still further. Also try alkali denat^n for step 1 :–

	7B	16C
Take 4 µl of B'87 (50 ng/µl)	4 µl	4 µl
+ 2.6 µl of { 6 µl A'102 ATR1B primer + 0.5 µl W139 3'Neo primer }	2.6 µl	2.6 µl
B'89 ALKALI: 300 µl 2 M NaOH, 700 µl ddW + 3.3 µl 0.6 M NaOH (pH check OK by paper)	3.3 µl	3.3 µl
let stand 5 minutes		
B'89 ACID (red): 300 µl 2 M HCl, 200 µl 1 M Tris pH 8, 500 µl ddW + 3.3 µl { 0.6 M HCl, 0.2 M Tris HCl pH 8 }	3.3 µl	3.3 µl
Sat at room temp. for several hours		
B'89B	(3.2 µl)	(3.2 µl)
B'89C		

Use 6 µl in ± Pfu with Special Mix n=5

ddW	64 µl	
20×PCR	6.25 µl	
4dNTPs	0.5 µl	
DMSO	12.5 µl	
Standard Taq	2 µl	0.4 µl / 25 µl
(5×17 µl	85 µl	

44 µl + ~0.1 µl Pfu

17 µl, 17 µl, 17 µl, 17 µl

B'89B (30) ~8 µl; B'89C (31) ~5 µl; B'89B (30') ~7 µl; B'89C (31') ~6 µl

Unequal

Regular Mix n = 14

dd W	252 µl	(n × 18 µl)
20×PCR	17.5 µl	(n × 1.25 µl)
4dNTP's	1.4 µl	(n × 0.1
DMSO	35 µl	(n × 2.5
W139 3'Neo primer	4.2 µl	(n × 0.3)
A'102 ATR1B primer	14 µl	(n × 1)
	322 µl	(14 × 23)
0.4 µl / 25 µl → +	5.6 µl Taq 1	

Rest 160 µl + ~0.3 µl Pfu

23 µl

1	2	3	4	5	6
C/2	C/4	C/8	B/2	B/4	B/8
1'	2'	3'	4'	5'	6'
50 ng	25 ng	12.5 ng	50 ng	25 ng	12.5 ng

AGT TAILCUFF DATA

Sheet12

COMPILATION

EXPERIMENT #1

SUMMARY OF ANTHOLOGY DATA

0 COPY ANIMALS																		
MOUSE	8667	8739																
EXP	1	3																
SEX	F	M																
BP	101	91																
HR	736	790																

EXPERIMENT #2

EXPERIMENT #3

1 COPY ANIMALS																		
MOUSE	8666	8668	8678	8679	8680	8689	8687	8688	8725	8726	8740	8741	8771					
EXP	1	1	2	2	2	2	2	2	3	3	3	3	3					
SEX	F	F	F	F	F	F	F	F	M	M	M	M	M					
BP	125	138	106	96	100	108	108	105	105	119	112	104	116					
HR	700	750	705	689	707	663	745	764	788	737	777	791	748					
	MEAN 1	131.5	725				MEAN 2	103.8333	712.1667			MEAN 3	111.2	768.2				
	STDEVP 1	6.5	25				STDEVP 2	4.412734	4.346135			STDEVP 3	5.912698	21.77522				
	SE 1	4.596194	17.67767				SE 2	1.801491	1.774302			SE 3	2.644239	10.88761				

2 COPY ANIMALS																		
MOUSE	9204	9206	9248	9256	8690	8738	8753	8754	8776	8777								
EXP	1	1	2	2	2	3	3	3	3	3								
SEX	F	F	F	F	F	M	M	M	M	M								
BP	129	123	132	99	82	125	133	112	120	126								
HR	701	657	683	712	740	716	815	743	774	769								
	MEAN 1	126	679	MEAN 2	104.3333	711.6667			MEAN 3	123.2	763.4							
	STDEVP 1	3	22	STDEVP 2	20.75786	23.27135			STDEVP 3	6.968501	33.07325							
	SE 1	2.12132	15.55635	SE 2	11.98456	13.43572			SE 3	3.116408	16.53663							

3 OR 4 COPY ANIMALS																		
MOUSE	8657	9247	8646	9253	9200	9250	9199	8647	9254	9249	9255							
EXP	1	2	2	2	2	2	2	2	2	2	2							
SEX	F	F	F	F	F	F	F	F	F	F	F							
BP	171	118	97	106	124	124	109.3	134	122	131	140							
HR	704	716	663	730	690	704	711	687	740	700	707							
										MEAN 2	120.53	704.8						
										STDEVP 2	12.62411	13.27725						
										SE 2	3.992093	4.425751						

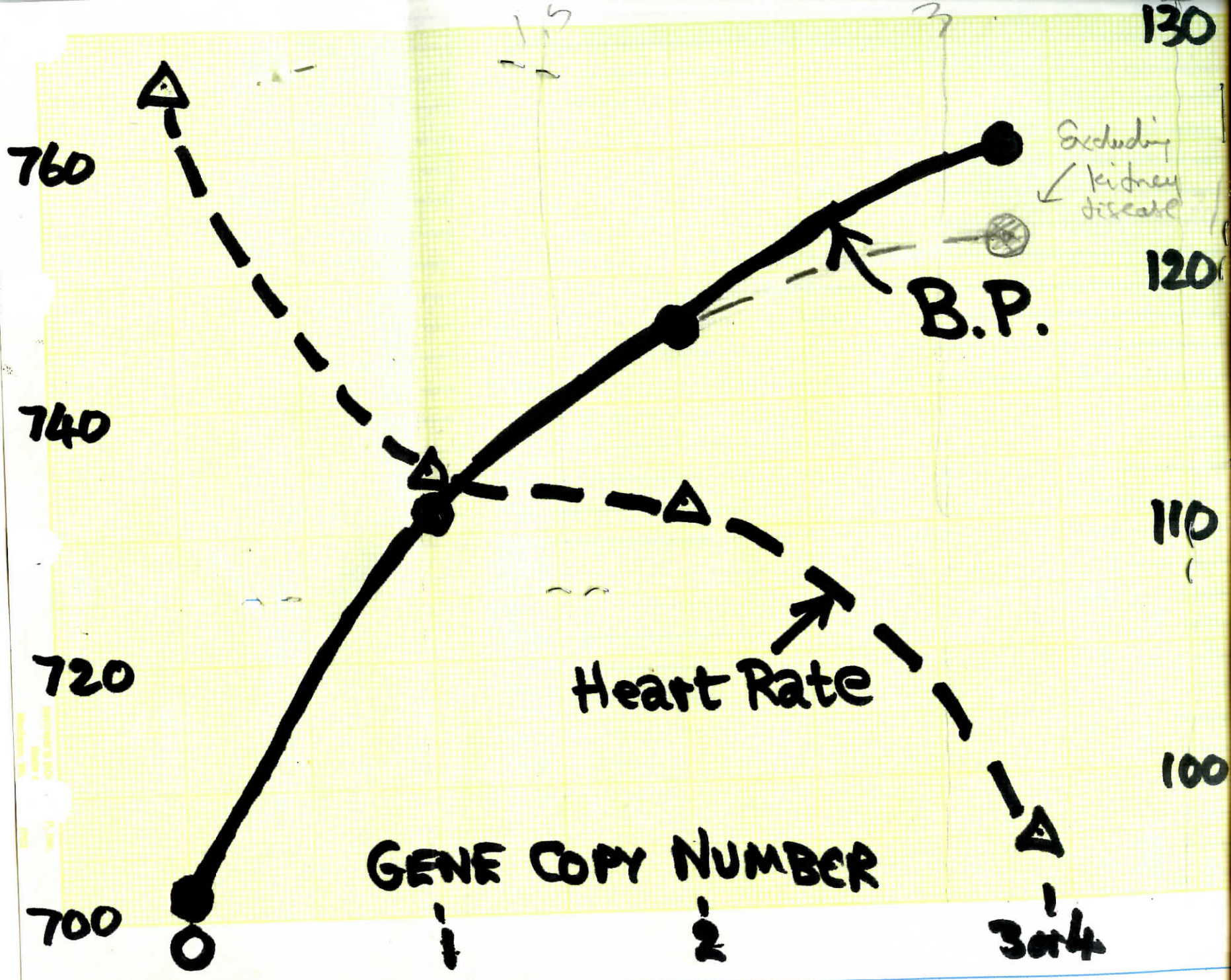
	MEAN AL	TDEVP A	SE ALL	RANK
	96	5	3.535534	#4
	763	27	19.09188	#1
	110.9231	10.83059	3.003866	#3
	735.6923	38.48153	10.67286	#2
	118.1	15.4172	4.875346	#2
	731	44.54211	14.08545	#3
	125.1182	18.8519	5.684062	#1
	704.7273	19.88645	5.995992	#4

1205 ended in 86

704.8 late

May 9th, 1994

9 Kidney disease


760
740
720
700
130
120
110
100
B.P.
Excluding kidney disease
Heart Rate
GENE COPY NUMBER
0
1
2
3 or 4

5' note: D8Mit56 left 3'

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

24
50%

5' note: D8Mit56 Right 3'

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

```
L34        D8Mit14                                          Tue 27 Apr 1993
MAP POSITION: 64.9 cM from top of Chr8 (Framework)
PRIMERS: TTTTCACACTCACGTGTGCG            GTCTCTCCTTCCTGGCGCTG
PRODUCT: 143 bp
GENOTYPE: HBABBAHHAHBAHHHHHHBA-AB BAHABBABBBBHHHAHBHHHHHH (rank 1)
SEQUENCE:     10         20         30         40         50         60
   0 GGGTGCTGGT GGAGNCCTCG NATACCCGGG NGATCTAAGA GGCTCTTCTA GACTTTGTAG
  60 GTACCTGTTT TCACACTCAC GTGTGCGTAC AGACTGAAAC ACACACACAC ACACACACAC
 120 ACACACACAA TTAGAAATAA TACTGAAAAA CAGAACAGGA AGTTGGCTGA AGTATACGTG
 180 CGTGTTACCC CAGCGCCAGG AAGGAGAGAC GGTGGATCAT CCATGCACAC GGCCCCATGA
 240 TAAAATATCA NGCTTATGGA ANGNGTTCAG TTTTNCTAAA CCACTGGGNN NATTTCAGTC
 300 TATGTGCTTG TGTCTGTANT AGAAGGNNGT NANTATCAAC TAACTAAAGA TTTCTTGTTG
 360 TTTAAAAAAG GNGGNANGGN GNCCTGGGGT CAGAACTGCA TGTACAANAG CCCATTNTCT
 420 CCAGNGACTN TCAANCCCCA AGNTGCCCTA GCATNGCCAN NGTTTTGAGG ATTCCTCTNA
 480 GAGTCCGCCC TTNNGNCNTT NANGGTTTGG CGTANTNGTG NGTGCATANT TTTTTCCTGT
 540 GTAAATTTGT TNTNNGTNNN ANTTTNCACA NNCCNTNNGG GCCCGAGAGT TNNNGTNTGG
 600 NGC
ALLELES:    ob cast  spr    a   b6  c3h  dba balb  akr  non  nod   lp
           142  155  130  167  142  138  142  167  138  142  138  167

MTH227     D8Mit148                                         Thu  4 Nov 1993
MAP POSITION: 64.9 cM from top of Chr8 (Framework)
PRIMERS: CATCTCCCCCTCACCTCATA            TGTTAGCAATATGGATTTTAGAGCC
PRODUCT: 238 bp
GENOTYPE: HBABBAHHAHBAHHHHHHBAAAB BAHABBABBBBHHHAHBHHHHHH (rank 1)
SEQUENCE:     10         20         30         40         50         60
   0 ACACCCACAT CTCCCCCTCA CCTCATATCT ACACCTACAC ACACACACAC ACACACCTGC
  60 ACATGTACAC ACACACACCT GCACATGTAC ACACACACAC ACACACACCT GCACATGTAC
 120 ACACACACAC ACACACACAC ACACCTGTAC ATGTACACAC ATAGACACAC ACACACACAC
 180 ACACACACAC ACACACACAC TTNGTAGATC TTGAGTATGA GGCTCTAAAA TCCATATTGC
 240 TAACAGGAGA TAGCTCTG
ALLELES:    ob cast  spr    a   b6  c3h  dba balb  akr  non  nod   lp
           239  227  369  239  239  243  239  239  243  239  239  227

M77        D8Mit13                                          Tue 27 Apr 1993
MAP POSITION: 64.9 cM from top of Chr8 (Framework)
PRIMERS: CCTCTCTCCAGCCCTGTAAG            AACGTTTGTGCTAAGTGGCC
PRODUCT: 98 bp
GENOTYPE: HBABBAHHAHBAHHHHHHBAAAB BAHABBABBBBHHHAHBHHHHHH (rank 1)
SEQUENCE:     10         20         30         40         50         60
   0 TTNAAGCNNG GTCCCGGGGA TCTCATTATG GATGGNTGTG AGCCACCATG TGTAGGCTGG
  60 GAATTGAACT CAGGACCTTT GGAAGAGCAG TCAGTGCTCT TAACCACTGA GCCTCTCTCC
 120 AGCCCTGTAA GAATATTTCT TACACACACA CACACACACA CACACACACA CACACGGAGG
 180 GGGAGAATTG GCCACTTAGC ACAAACGTTG ATAAAGCAGA GGCTACACTA CGTACTACAC
 240 GGGCATAACC ACGGCACCCA CAGGATGGAC GGTGATTATC ACAAAGCTCT TACACTTCAC
 300 AGGAAGTGTG GGCTGTGATG ATGCAGAGAC CTATGCTAGC TTCCCAGAGC CACCAGACAA
 360 TGATGGACCT CACAAGACGT TATTATAGAG AAGCGCCAAA AGGNACCACC CAGAT
ALLELES:    ob cast  spr    a   b6  c3h  dba balb  akr  non  nod   lp
           98  114  114   98   98   91   98  105   94   98   98  108
^L                          MAP BASE QUERY REPORT
```

129 is like other

129 is like this

Genome_Database @ genome.wi.mit.edu "help"

Bill Dietrich
(617) 252 1915

Thurs. June 2nd

O.S.

Genotyping 2/1 v. 2/2

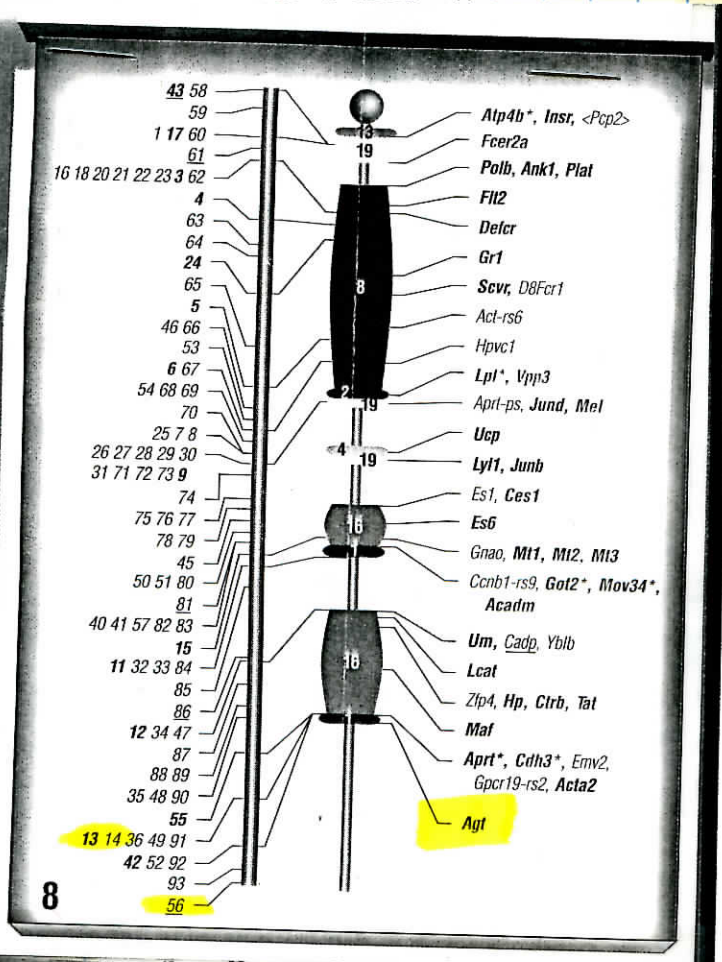
Kim has ~~looked~~ for ~~Hetlet~~ for any markers in Agt gene & none found. Sylvie & Madeline tested quantitative Southerns for genotyping 2/1 v. 2/2. Very unsatisfactory.
Better logic is that in F2 the heterozygotes are always B6 and 129-derived, the homozygotes are B6 (normal) or 129 (doubly-mutant). ∴ any marker closely linked to Agt can be used for genotyping.
Fetch's lots with D8 Mit13 and Mit 14 done by Ken look promising (at 64.9 cM). Agt is at ~70 cM. D8 Mit 156 is at 70.5 cM & so c'd be better in theory. Text D8 Mit 156 and 56, using primers to left. Note lp is fairly close to 129 as Fetch's genealogy, & 13 & 14 give predicted (lp) sizes.

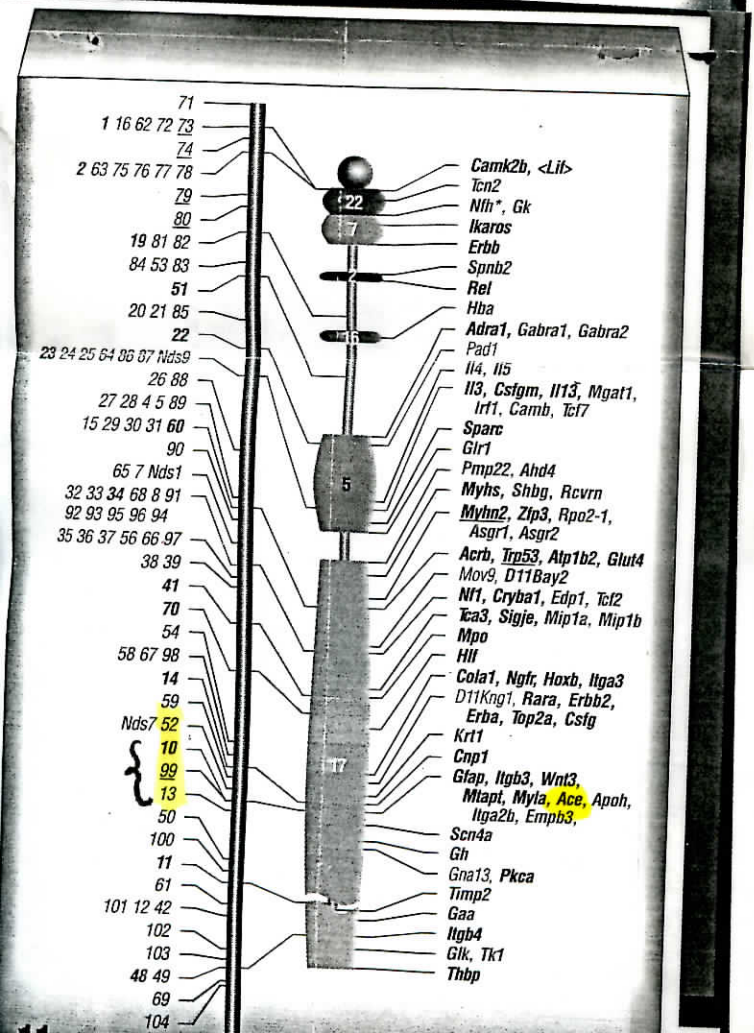
Mon 5 Oct 1992

```
B740        D8Mit56
MAP POSITION: 70.5 cM from top of Chr8 (Placement)
PRIMERS: ACACTCAGAGACCATGAGTACACC        GAGTTCACTACCCACAAGTCTCC
PRODUCT: 162 bp
GENOTYPE: HBAHHAHHAHBAHHHHAHBAAH- BABABBABBBBHHHAABHHHHHH (rank 1)
SEQUENCE:     10         20         30         40         50         60
   0 CCGCTCCTGA GACTACTTCA GACACTCAGA GACCATGAGT ACACCACTAA GAGGAGCAAG
  60 TAAATGGTGG AGAAACAGAA GAAAAAGAGA AACAGGAGGA TATGGGCGCG CACAAACACA
 120 CACACACACA CACACACACA CGGCATAACT GGAGACTTGT GGAGACTTGT GGGTAGTGAA
 180 CTCAATTAAT TAGGCTTAAT TTGAGTAGTT
ALLELES:    ob cast  spr   a   b6  c3h  dba balb  akr  non  nod   lp
            160  184  152 164 160 164  182  164  164  160  180  182
                                                                    4
                          MAP BASE QUERY REPORT
Thu Jun  2 13:50:25 EDT
```

Mon 15 Nov 1993

```
MT1329      D8Mit156
MAP POSITION: 70.5 cM from top of Chr8 (Placement)
PRIMERS: ATTCTGCAATGCAAATACATACA        AACCAATGGCCTCCAGAAG
PRODUCT: 139 bp
GENOTYPE: HBAHHAHHAHBAHH-HAHBAAHB BABABBABBBBHHHAABHHHHHH (rank 1)
SEQUENCE:     10         20         30         40         50         60
   0 ATTCTGCAAT GCAAATACAT ACACACACAC ACATACACAC ACACACACAC ACAGAGAGAG
  60 AGAGAGAGAG AGAGAGAGAG AGAGAGAACT TGTGGTTTCA CTGACCTTTC AAGTGGTGGC
 120 CTTCTGGAGG CCATTGGTTC CAGTCACAAA TATAGATACT ATTGTGATAA GCAAGTTAAA
 180 TACAAATGAG TACCCCAAGA GCCTAAGAAG TTATCTTGTT AGAACCTCCA GGNCTCCTTT
 240 TATTTATGGC ATCTAGTATA GGAAAGNCTC TCAACTAACC TGAGAAGTAG GTCTCAAGAA
 300 ACATGCTGGT ATCAAGGAAA TGAGTGCAGA TGTATGAGTA AT
ALLELES:    ob cast  spr   a   b6  c3h  dba balb  akr  non  nod   lp
            140  158  150 154 140 154  154  154  154  140  154  152
```





The chart shows a current genetic map of the mouse that provides a marker every 0.6 centimorgans (cM) on average, which is roughly equivalent to every 1.1 megabases. The genetic markers are of two types: 1518 simple sequence length polymorphisms (SSLPs), shown on the left, and 1098 gene-based loci, shown on the right.

SSLP Map. The SSLP map consists of loci defined by polymerase chain reaction (PCR) assays, each involving a specific pair of PCR primers flanking the site of a short repeat sequence of variable length — in most cases, $(CA)_n$. These markers tend to be highly polymorphic among inbred laboratory strains, making them especially useful for genotyping intraspecies crosses. Most were based on anonymous (random) sequences, although about one-tenth were taken from repeats found in known genes. The SSLP markers shown were all genotyped in a single interspecies F_2 cross [W. Dietrich *et al.*, *Genetics*, **131**,423 (1992)]. The data were subjected to a mathematical error-checking procedure [(S.E. Lincoln and E.S. Lander, *Genomics* **14**, 604 (1992)], with the result that the relative positions of the loci were supported by a likelihood ratio of 1000:1, except for underlined loci, for which the ratio was >10:1. For nearly 98% of the markers, which were developed at the Whitehead Institute/MIT Center for Genome Research (WI-CGR), Cambridge, MA, formal locus names have been abbreviated. For example, D17Mit3 is shown as 3 on chromosome 17. The remaining SSLPs were developed elsewhere but were also genotyped in the same cross. For these loci, the symbol of the originating laboratory was retained (For example, D4Nds1 is abbreviated Nds1).

Gene-Based Map. The gene-based map is primarily defined by cloned gene probes that detect restriction fragment length polymorphisms (RFLPs). This map is especially valuable for comparative mapping between the mouse and human genomes. The framework for the map is the Frederick interspecies backcross map [N.G. Copeland and N.A. Jenkins, *Trends Genet.* **7**,113 (1991)], shown in *black type*. Because these loci were all mapped in a single backcross and subjected to mathematical error-checking, the orders of loci were supported by a likelihood ratio of 1000:1, except for underlined loci for which the ratio was >10:1. Loci shown in ***red*** (■) consist of additional genes and anonymous DNA segments mapped in human and mouse that were not mapped in Frederick, but were reported in the 1993 Mouse Chromosome Committee Reports (*Mammalian Genome*, in press); the positions of these genes were inferred on the basis of mapping information in these committee reports. Loci in brackets <> were used to align the maps with respect to the centromere. The position of the centromere is indicated by a circle at the top of each chromosome. Chromosome lengths were drawn to scale on the basis of a 1600-cM genetic length. A break in the distal region of chromosome 11 indicates that this chromosome is slightly longer than predicted by ≈ 1600-cM map.

Integration of Maps. The two genetic maps were integrated by mapping approximately 250 of the SSLPs from the WI-CGR in the Frederick interspecies backcross. These SSLPs are indicated in ***green*** (■), with lines connecting their locations in the two crosses.

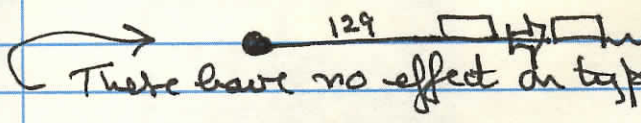
Mouse-Human Homology. The colored segments within each chromosome map indicate known regions of synteny or linkage conservation between the mouse and human genomes. The correspondence is based on those mouse genes whose human homologs have been mapped in the human genome; these loci are indicated in ***bold***. For loci mapping near the boundary of two human homology segments, those loci mapping to the proximal segment are marked with an asterisk.

Table. The table included in the chart contains a representative list of

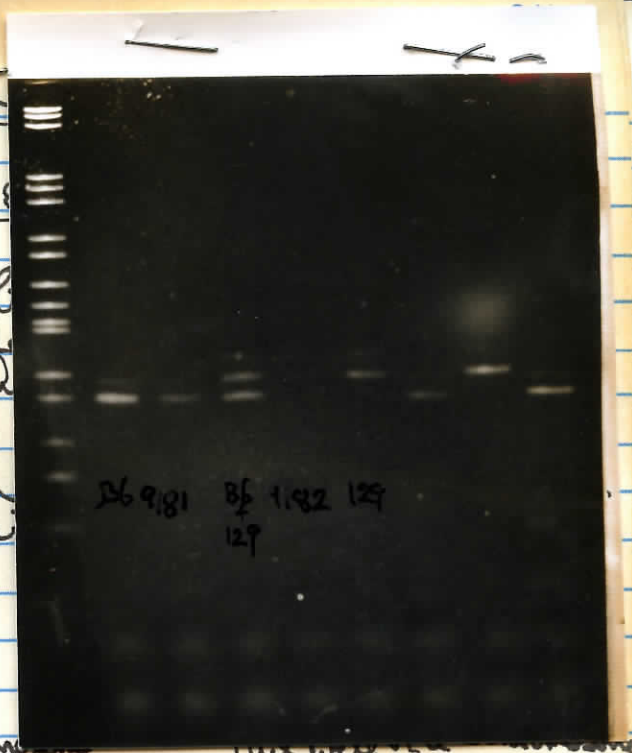
Fri. Jun

MIT 13/14

Kim tested 13 and 14 – (
Recombinants will arise d
~~from~~ Agt. Recombinant d

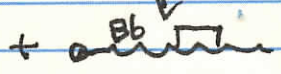


These have no effect on typi

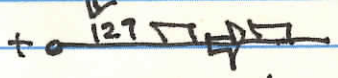


This will be a 1 chromo
but will M13 type as 129

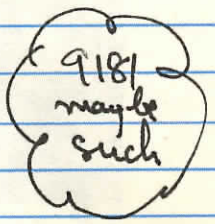
This will be a
but will M13 type as B6

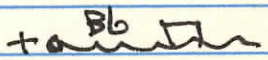


This animal is 1/1 but will M13 type as 2/1. No problem because it will be Neo⁻ (will measure map distance)



This animal is 2/1 but will M13 type as 2/2. It will ∴ score incorrectly.

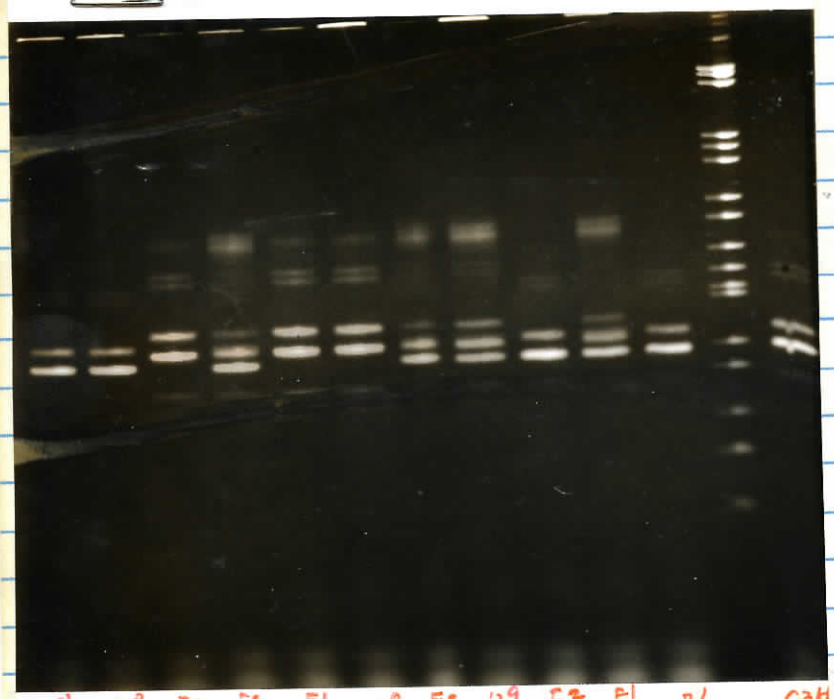




This animal is 2/1 but will M13 type as 1/1. No problem because it will be Neo⁺ (will measure map distance)

This animal is 2/2 but will M13 type as 2/1. It will ∴ score incorrectly

So 50% of recombinants will be recognized & cause no error
Other 50% will not be recognized & will blur the edges of 2/1 & 2/2



MIT 56 looks good, also
but 56 is not good
(c.f. mp. of primer)

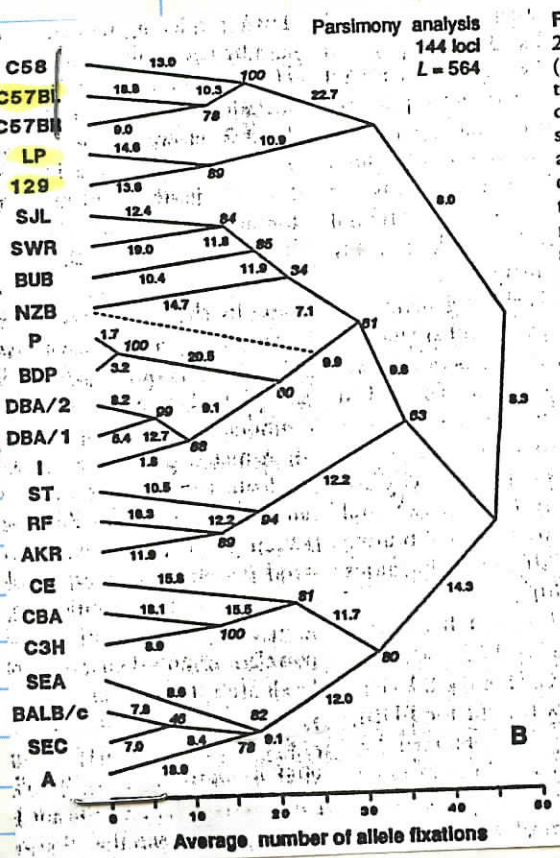
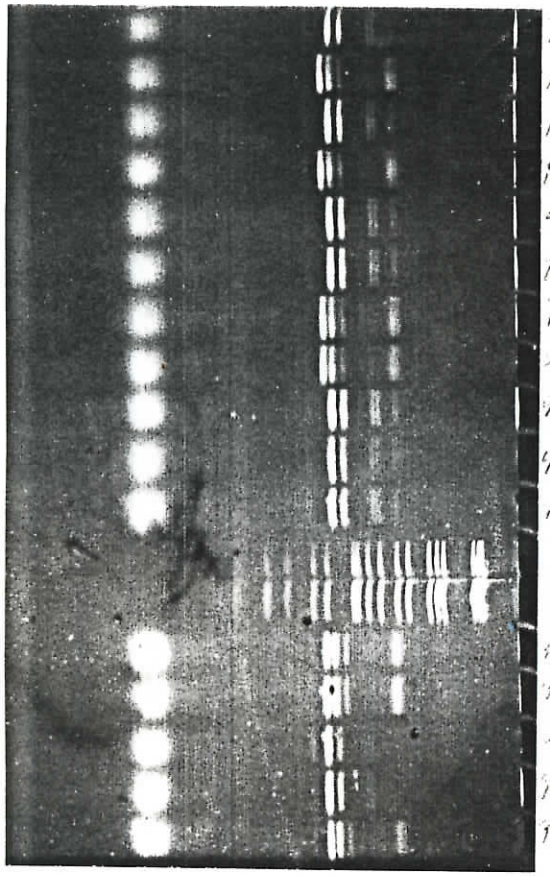
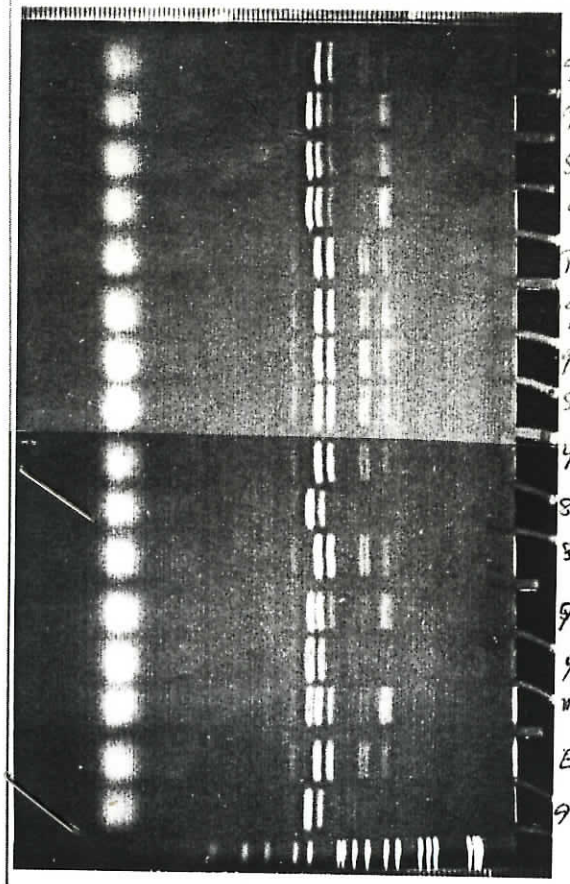


Fig. 1. (**A**) Genealogy of 24 inbred strains of mice (*10–12*). Black dots refer to crosses made in production of a particular strain. (**B**) Parsimony analysis of 144 single locus genotypes (rather than a genetic distance matrix) for 24 inbred strains of mice. Phylogenetic trees representing estimated relationships among strains were produced by using the parsimony algorithm (*15*). The length of the tree (*L*) reflects a total of 565 allele fixations. The branch lengths are in allele fixations (arabic numbers). The numbers in italics reflect the number (out of 100 resamplings of the data) that give the same clade (*16*). Nonintegral numbers of substitutions (allele fixations) arise from averaging over all possible parsimonious solutions for the tree. Dotted lines represent alternative but equally parsimonious joinings of taxa.


9245
9255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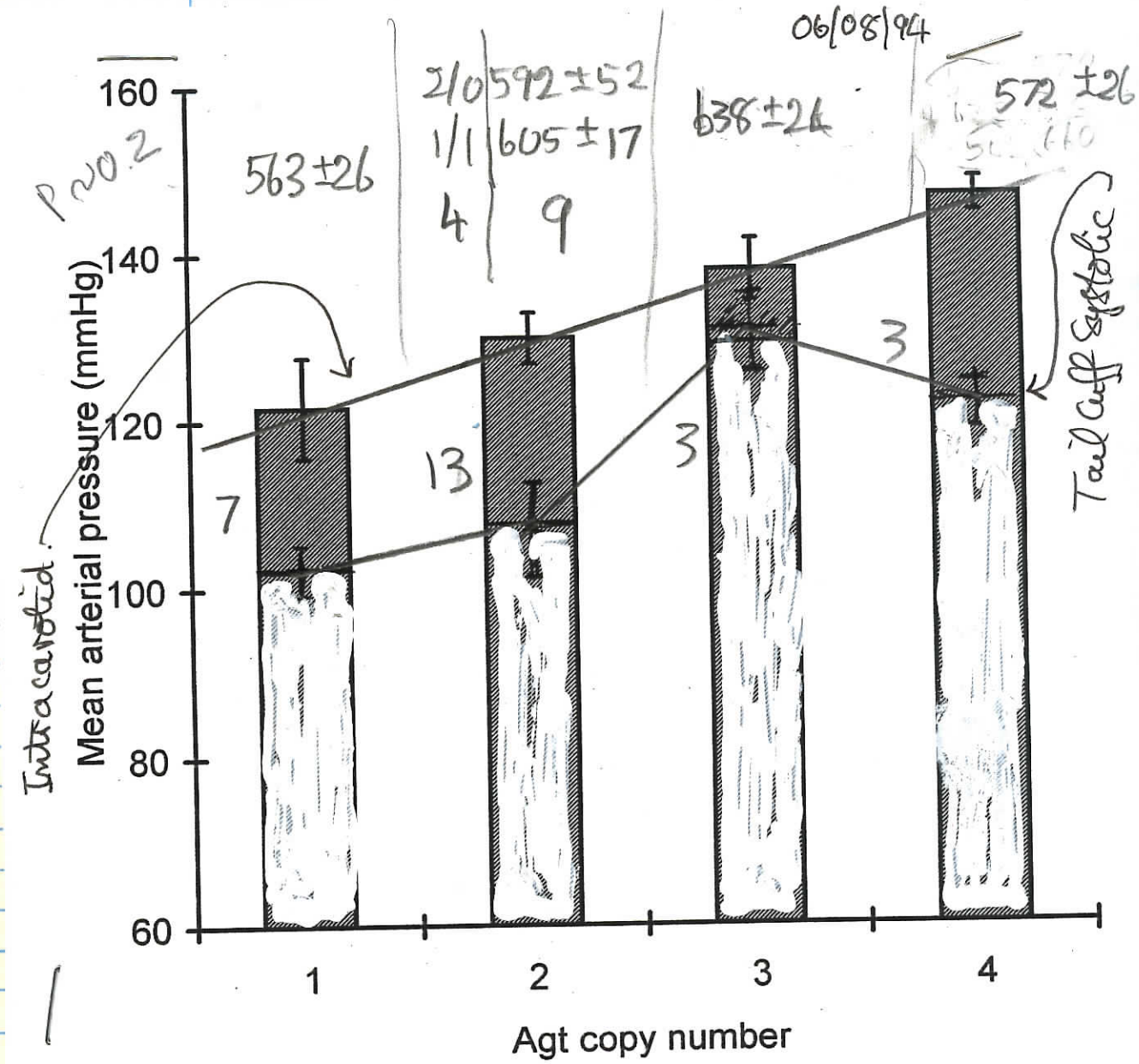
9257
9262
9152
9151
mix
E14
9310



Resting/Conscious

Data on crossing over (absence)

	2/1	2/2	total
37-120	2	3	5
37-112	6	5	11
37-94	7	3	10
37-76	15	6	21
37-66	22	6	28
37-63	29	10	39
37-58	10	13	23
	91	46	137
	B6/129	129/129	

Mit 56.

Absence of any Neo$^+$ B6$^+$ 129 shows no X.O. in ~91 meioses.

11/25/94

(2) ~~AGT Neo AGT 56 Long (129)~~

(1) ~~AGT 56 Short (B6)~~

From Bob Reddick — Oliver

Rapid Publication

Recombinant Platelet-derived Growth Factor B Gene Expression in Porcine Arteries Induces Intimal Hyperplasia In Vivo

Elizabeth G. Nabel,* Zhiyong Yang,* Susanne Liptay,* Hong San,* David Gordon,* Christian C. Haudenschild,‡ and Gary J. Nabel*

**Departments of Internal Medicine, Biological Chemistry, and Pathology, Howard Hughes Medical Institute, University of Michigan, Ann Arbor, Michigan 48109; and ‡Jerome H. Holland Laboratory, American Red Cross, Rockville, Maryland 20855*

Abstract

Platelet-derived growth factor (PDGF) B chain induces cell proliferation in vitro and is associated with arterial lesions that cause cardiovascular disease. However, it has been difficult to document the biological response to PDGF B gene expression in arteries in vivo. To determine the biologic effects of this growth factor in vivo, we have introduced an eukaryotic expression vector plasmid encoding recombinant PDGF B by direct gene transfer into porcine iliofemoral arteries using DNA liposome complexes. The presence of PDGF B plasmid DNA and expression of recombinant mRNA were confirmed by polymerase chain reaction analysis, and recombinant PDGF protein was demonstrated by immunohistochemistry. Intimal thickening was observed in porcine arteries 21 days following transfection with the recombinant PDGF B gene compared with arteries transduced with a control gene, E. coli β-galactosidase. An eightfold increase in intimal to medial ratio was present in PDGF B gene transfected arteries compared with control transfected arteries ($P = 0.001$). This study suggests that expression of a recombinant PDGF B gene in vivo can play a role in the induction of intimal hyperplasia, which can lead to cardiovascular diseases. (*J. Clin. Invest.* 1993. 91:1822–1829.) Key words: gene transfer • gene expression • platelet-derived growth factor • cellular proliferation • liposomes

Introduction

Multiple growth factors stimulate vascular smooth muscle cell proliferation in vitro, including the dimeric forms of PDGF. PDGF BB is a potent smooth muscle cell mitogen (1, 2) that may also act as a smooth muscle chemoattractant (3–5). In vivo, the role of the PDGF B gene in tissue injury and atherosclerosis has been difficult to address. This difficulty arises from the complexity of cellular and protein interactions in vivo, their paracrine and autocrine nature, and the cascade of events that can be induced by experimental manipulations. It is often difficult to determine whether a specific gene product induces intimal hyperplasia directly or indirectly through other gene products synthesized in response to injury. Recently, it has become possible to express recombinant genes in vivo by direct gene transfer utilizing retroviral vectors or DNA liposome complexes (6–9). Direct gene transfer permits the delivery of a specific recombinant gene into vascular cells at specific sites in vivo and affords the opportunity to determine the effects of a specific gene product in the arterial wall (10). To define the function of PDGF B within arteries, we have directly introduced the recombinant gene in porcine arteries in vivo and have shown that expression of recombinant PDGF B can cause intimal hyperplasia. These data suggest that expression of PDGF B can induce vascular lesions relevant to the pathogenesis of atherosclerosis and restenosis.

Methods

Plasmids and cell transfection. The PDGF B expression vector used for in vitro and in vivo experiments was prepared by ligating the SalI-XbaI fragment of the v-sis gene into the XhoI-BamHI cloning site of the pSVL vector (11) (Pharmacia, LKB Biotechnology, Piscataway, NJ). This vector utilizes the SV40 late promoter to regulate expression of the PDGF B gene. Primary porcine endothelial cell cultures were established as previously described (12) to test expression of the PDGF B vector. The pSVL/PDGF B plasmid expression vector was transfected into porcine endothelial cells using 12 μg of Lipofectin® (Bethesda Research Laboratories, Gaithersburg, MD) and 5 μg of DNA (stock concentration > 1 mg/ml) (13). Culture transduced endothelial cells were assayed for secretion of recombinant PDGF BB supernatants (10%) with a colorimetric proliferation assay (14) using NIH 3T3 cells, and a fourfold or greater increase in proliferative activity was observed. No evidence of cell transformation was observed in endothelial or vascular smooth muscle cells stably transduced with this vector. The E. coli β-galactosidase expression vector was prepared by cloning the β-galactosidase gene into the pDOL vector, which was derived from the Moloney murine leukemia virus (Mo-MuLV) (15). The wild type Mo-MuLV LTR provided the promoter for the β-galactosidase gene.

Gene transfer in vivo. In vivo arterial gene transfer was performed in 15 pigs, 9 with the recombinant PDGF B gene and 6 with a control reporter gene, E. coli β-galactosidase. Double balloon catheters were positioned in the iliofemoral arteries as previously described (6). The arterial segment was rinsed with 5 ml saline and 5 ml Opti-MEM to clear the vessel of blood. Approximately 10 min before the insertion of the catheter, the DNA liposome conjugates were prepared. 5 μl lipofectin was diluted into 0.2 ml Opti-MEM at room temperature, and 2–5 μg plasmid DNA (stock concentration > 1 mg/ml) was added and mixed by gentle tapping. The solution remained at room temperature for 5–10 min, and 0.5 ml Opti-MEM was added to the DNA liposome

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Mon. June 20th

On atherosclerotic plaques

N.M.'s data on log distribution of plaque sizes, combined with published work on clonal nature of atherosclerotic plaques, suggests, as published, that they are partially transformed (c.f. anaplastic lesions, pre-cancer). If so they should grow better in culture.
Left with Ed Sheseley, the growth of F2 E^+/E^+ versus E^-/E^- vascular endothelial cell cultures.

Wed. June 22nd

Nobuyo comments that the (transformed) cells are first macrophages, then altered muscle cells, so we may have to look for strange outgrowths -
O.S. - anyway transplantability is what I'm looking for at first.

Thurs. June 23rd (O. & R.'s birthday 69!)

PGF B c'd be a candidate (See Nabel et al. J.C.I 91 1822 (1993) - brought by Bob Redfield)

Fri. June 24th

- or its receptor!

Mon. June 24th

With Nobuyo

$$V = \frac{V_{max}[S]}{K_m + [S]}$$

$$\therefore \frac{V}{V_{max}} = \frac{[S]/S_{0.5}}{1 + [S]/S_{0.5}} \qquad (S_{0.5} = K_m)$$

$$\text{or } y = \frac{x}{1+x}$$

$$\therefore \frac{dy}{dx} = \frac{1}{1+x} - \frac{x}{(1+x)^2}$$

As $x \to 0$ $\frac{dy}{dx} \to 1$

as $x \to \infty$ $\frac{dy}{dx} \to 0$

At $x = 1$ $\frac{dy}{dx} = \frac{1}{2} - \frac{1}{4} = \frac{1}{4}$

$\therefore$ slope at $[S] = K_m$ is 4 × slope at $[S] = K_m/100$

Human

Mouse

Hence mo. is ~4× more sensitive to [AGT] than human

Shingles!!

Let's see what happens when the clearance (receptor) varies

$2p$ = Utilization + Clearance = Constant

∴ if clearance capability decreases the concentration of AGT will ↑ until new utilization & new clearance are at equilibrium.

Consider a heterozygote for disruption of clearance gene. Let new level of AGT be $r \times$ old.

Assume util.^{n} is $\propto$ [AGT]

Then $U_{100} + C_{100} = r \cdot U_{100} + r \cdot \frac{C_{100}}{2}$

But $U_{100} = 0.3 \times C_{100}$

$\therefore \; 1.3 C_{100} = r(0.3 + 0.5) C_{100}$

And $r = 1.3/0.8 = 1.63$

Thus we can predict that [AGT] will increase 1.63 fold if clearance of AGT decreases to half.

This type of reasoning will apply to the ANP clearance receptor. It may apply to the RIA and RIB experiments, since the clearance of ATII will be changed, but less likely; more likely renin will ↑ & [AGT] will go down and ATII will ↑.

Sunday July 31

Some more equations (see B'78)

Let's see what solutions follow if util^n of AGT → AT^{II} & thence bp. ↑ with ↑ in gene copy.

∴ Let U (utilization) be proportional to steady state bp, with bp = 100 mm in the 1/1 animal and with 8 mm Hg change / copy number

Then

1/1 $$2p = U_{100} + C_{100} \qquad (1)$$

1/0 $$p = 0.92\,U_{100} + 0.35\,C_{100} \qquad (2)$$

2/1 $$2px + p = 1.08\,U_{100} + 1.24\,C_{100} \qquad (3)$$

Substituting (2) into (1) gives

$$1.84\,U_{100} + 0.7\,C_{100} = U_{100} + C_{100}$$

$$\text{or } 0.84\,U_{100} = 0.3\,C_{100}$$

$$\text{and } U_{100} = 0.357\,C_{100} \quad \text{Was } 0.3$$

And $$p = \frac{1.357}{2}\,C_{100} = 0.679\,C_{100} \quad \text{Was } 0.65$$

And $$2px = \underset{(0.386)}{1.08 \times 0.357}\,C_{100} + 1.24\,C_{100} - 0.679\,C_{100}$$

$$= 0.947\,C_{100}$$

$$x = \frac{0.947\,C_{100}}{2 \times 0.679\,C_{100}} = 0.697 \quad \text{Was } 0.685$$

Thus variable utilization does not render the system invalid.

(Cont^d)

If not, will it be purified later by gel ...

5' 08277 note: CR 5-13 1LEFT 3'

GCT	CAA	AAG	ATC	GAG	
GTG	CTT	GTG	C		

25
52%

5' 08278 note: CR 140-147 1MIDLEFT 3'

GAG	ATG	ATG	CTC	GCT	
CTG	TTT	CG			

23
52%

5' 08279 note: CR 148-141 1MIDRIGHT 3'

TGG	CGA	AAC	AGA	GCG	
AGC	ATC	AT			

23
52%

08280 5' 1Right note: CR 209-140 ← Mislabelled Sh'd be 202 3'

CTG	CCT	TGG	ATG	TAG	
CGC	ACT	AT			

23
52%

Overall will be 61[illegible] bp 5 portion will be 4[illegible] bp 3 will be 2[illegible] bp

A different "middle" could be in the rat territory

(+125)

E Y S H L T R V A P

or better (+109)

M L S A G A L A A G F

— but try mo. sequences first.

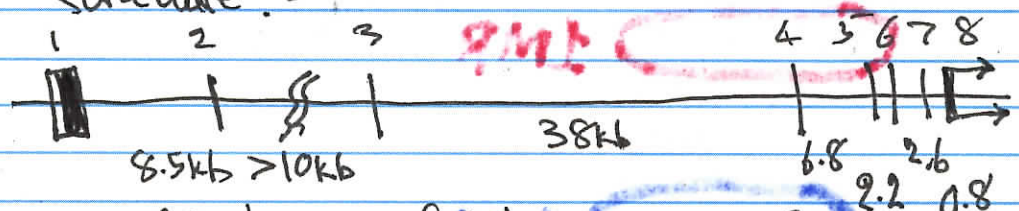
Tues. Aug. 2nd

ANP Clearance Receptor (CR)

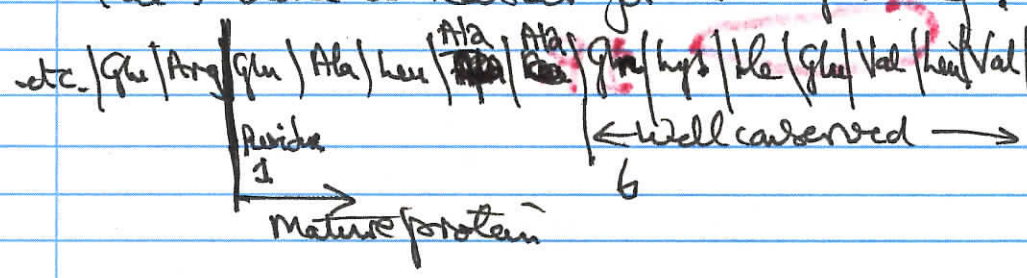
In view of importance of clearance in modulating concentrations, I will try to alter ↑ & ↓, the ANP Clearance Receptor.

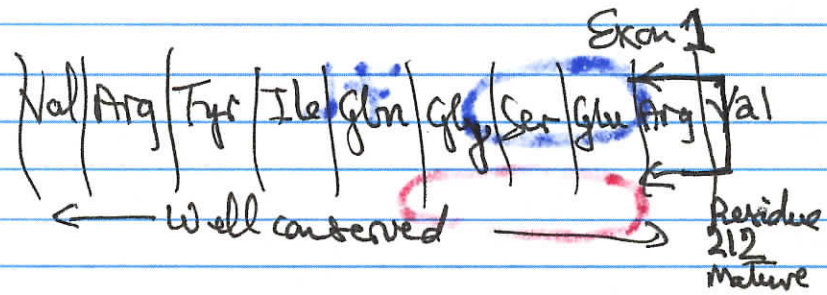
Sequence data on Bovine ANP Receptor (Type C) from Sakaki et al. J. BC. 266 11122 (1991) & from Fuller et al. J. B. C. 263 9395 (1988) & on rat & mouse (cDNA) from David Lowe (unpublished)

Structure: —



Since the 3' exon is of indeterminate length, it is probably best to keep 5' short & 3' long. The 5' clone is easier for a beginning.





6 then 211 w'd be >600bp for 1 probe or 2 x ~300 for 2 probes

All these worked

ψ111 5' ACE promoter probe was ~1 kb w1 5' ACE intron 1 probe was ~1 kb

w16 Hart intron 3' ACE was ~480 bp ψ88 3' UT 3' ACE was ~270 bp (cont'd)

labelled B'108 &) etc. Taken up in 500µl TE 1XCIAA

Summary of these first primer pairs

Full name	Trivial Name	Expected length	Comments
CR 5-13	1 Left (exon 1 5') 1L	→ 615 bp	All coding sequence of mature protein from (where it) exon 1
CR 209-140	1 Right (exon 1 3') 1R		
CR 5-13	1 Left 1L	→ 434 bp	Uses the first available mouse data ∴ is first 2/3 of exon 1
CR 148-141	1 Midright 1MR (mid exon 1 mouse data)	May be contaminated with bench dirt	
CR 140-147	1 Mid left 1ML (mid exon 1 mouse data)	→ 210 bp	Mouse data only ∴ is the second 1/3 of exon 1
CR 209-140 ↑ slide 202	1 Right (exon 1 3') 1R		

Test with all three. Hope to use the 2/3 & 1/3 for double lifts

CR 433-440	7 left (exon 7 5' end) 7L	→ Uncertain - prob. ~1kb	Exon 7 intron 8 Exon 8
CR 488-481	8 Right (exon 8 3' end)		

Wed. Aug 3rd

3' end of ANP Clearance Receptor (CR)

The 3' end is complicated by the presence of an 3' untranslated region of unknown length, but

082 86
le: -
I: 23
52%

5' note: CR488-481 8 Right 3'

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

08287
le:
I: 24
50%

5' note: CR433-440 7 left 3'

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

length 25 GC 44% ← Not v. good

	G	K (481)	H	R	E	L	R	E	D (488)	S
or better	GGG	AAG	CAT	CGA	GAG	CTG	CGA	GAA	GAT	TCC
		CTT	GTA	GCT	CTC	GAC	GCT	CTT	CTA	AGG

length 23 GC 52% CR 488-481

G (433)	L	E	E	S	A	V	T (440)	G	I	V
GGC	CTA	GAA	GAA	TCT	GCA	GTG	ACA	GGA	ATC	GTT

length 24 GC 50% CR 433-440

← header →

Mature

```
                                  •                                                                   +1
rNPRC  -40  M R S L L L F T F S A C V L L A R A L L A G G A - - - - - S S G G G D T G P G N R R R E R E A L A A
hNPRC  -45  M P S L L V L T F S P C V L L G W A L L A G G T G G G G V G G G G G G A G I G G G R Q E R E A L P P
bNPRC  -41  M P S L L V L T F S A C V L L G W A L L A D C T G - - - - G G G S G G A G P G R G R R E R E A L P P
                                                                                            *
rNPRC    6  Q K I E V L V L L P R D D S Y L F S L A R V R P A I E Y A L R S V E G N G T G R K L L P P G T R F Q
hNPRC    6  Q K I E V L V L L P Q D D S Y L F S L T R V R P A I E Y A L R S V E G N G T G R R L L P P G T R F Q
bNPRC    6  Q K I E V L V L L P Q D D S Y L F S L A R V R P A I E Y A L R T V E G N A T G R R L L P A G T R F Q
```

Primer 1 Left

112

```
                          •                                                       •
rNPRC   56  V A Y E D S D C G N R A L F S L V D R V A A A R G A K P D L I L G P V C E Y A A A P V A R L A S H W
hNPRC   56  V A Y E D S D C G N R A L F S L V D R V A A A R G A K P D L I L G P V C E Y A A A P V A R L A S H W
bNPRC   56  V A Y E D S D C G N R A L F S L V D R V A A A R G A K P D L I L G P V C E Y A A A P V A R L A S H W
```

507

IMR2

1 mid Right

Primer

1 mid left

```
rNPRC  106  D L P M L S A G A L A A G F Q H K D T E Y S H L T R V A P A Y A K M G E M M L A L F R H H H W S R A
hNPRC  106  D L P M L S A G A L A A G F Q H K D S E Y S H L T R V A P A Y A K M G E M M L A L F R H H H W S R A
bNPRC  106  D L P M L S A G A L A A G F Q H K D T E Y S H L T R V A P S Y A K M G E M M L A L F R H H Q W S R A
```

182

```
                                    •
rNPRC  156  A L L Y S D D K L E R N C Y F T L E G V H E V F Q E E G L H T S A Y N F D E T K D L D L D D I V R Y
hNPRC  156  A L V Y S D D K L E R N C Y F T L E G V H E V F Q E E G L H T S I Y S F D E T K D L D L E D I V R N
bNPRC  156  V L V Y S D D K L E R N C F F T L E G V H E V F Q E E G L H T S A Y N F D E T K D L D L E D I V R H
```

Primer 1 Right

Exon 1 | 2

Exon*2 | Exon 3

```
                                •
rNPRC  206  I Q G S E R V V I M C A S G D T I R R I M L A V H R H G M T S G D Y A F F N I E L F N S S S Y G D G
hNPRC  206  I Q A S E R V V I M C A S S D T I R S I M L V A H R H G M T S G D Y A F F N I E L F N S S S Y G D G
bNPRC  206  I Q A S E R V V I M C A S S D T I R G I M L A A H R H G M T S G D Y A F F N I E L F N S S F Y G D G
```

Exon 2 | Exon 3

```
rNPRC  256  S W K R G D K H D F E A K Q A Y S S L Q T V T L L R T A K P E F E K F S M E V K S S V E K Q G L N E
hNPRC  256  S W K R G D K H D F E A K Q A Y S S L Q T V T L L R T V K P E F E K F S M E V K S S V E K Q G L N M
bNPRC  256  S W K R G D K H D F E A K Q A Y S S L Q T I T L L R T A K P E F E K F S M E V K S S V E K Q G L S E
```

Exon3 | Exon 4

* Exon4 | Exon 5

```
rNPRC  306  E D Y V N M F V E G F H D A I L L Y V L A L H E V L R A G Y S K K D G G K I I Q Q T W N R T F E G I
hNPRC  306  E D Y V N M F V E G F H D A I L L Y V L A L H E V L R A G Y S K K D G G K I I Q Q T W N R T F E G I
bNPRC  306  E D Y V N M F V E G F H D A I L L Y V L A L R E V L R A G Y S K K D G G K I I Q Q T W N R T F E G I
```

Exon 5 | Exon 6

```
rNPRC  356  A G Q V S I D A N G D R Y G D F S V V A M T D T E A G T Q E V I G D Y F G K E G R F K M R S N V K Y
hNPRC  356  A G Q V S I D A N G D R Y G D F S V I A M T D V E A G T Q E V I G D Y F G K E G R F E M R P N V K Y
bNPRC  356  A G Q V S I D A N G D R Y G D F S V I A M T D T E A G T Q E V I G D Y F G K E G R F E M R P N V K Y
```

Exon 6

Exon 7

```
                                                *       •     •
rNPRC  406  P W G S L K L R I D E T R I V E H T N S S P C K S C - G L E E S A V T G I V V G A L L G A G L L M A
hNPRC  406  P W G P L K L R I D E N R I V E H T N S S P C K S C - G L E E S A V T G I V V G A L L G A G L L M A
bNPRC  406  P W G P L K L R I D E T R M V E H T N S S P C K A S G G L E E S A V T G I V V G A L L G A G L L M A
```

7 left

Exon 7 | Exon 8

```
rNPRC  455  F Y F F R K K Y R I T I E R R N H Q E E S N I G K H R E L R E D S I R S H F S V A
hNPRC  455  F Y F F R K K Y R I T I E R R T Q Q E E S N L G K H R E L R E D S I R S H F S V A
bNPRC  456  F Y F F R K K Y R I T I E R R N Q Q E E S N V G K H R E L R E D S I R S H F S V A
```

8 Right

From David Lowe

Fri Aug 20 14:55:03 1993
/home/sprite/va/cardio/sandberg/aa.out


```
ratNPRC   1 GAATTCCGCTTGCTGGCAACAGGATGCGGTCCCTGCTGCTGTTCACTTTC

ratNPRC  51 TCGGCGTGCGTGCTGCTGGCCCGGGCGCTGCTGGCTGGCGGTGCGAGTAG

ratNPRC 101 TGGCGGTGGGGACACCGGGCCAGGCAACAGGCGCCGGGAGAGAGAGGCGC

ratNPRC 151 TGGCGGCTCAAAAGATCGAGGTGCTTGTGCTATTGCCCCGGGACGATTCG

ratNPRC 201 TACTTGTTCTCGCTGGCCCGGGTGAGGCCGGCCATCGAGTACGCGCTGCG

ratNPRC 251 TAGCGTGGAGGGCAATGGCACCGGCAGGAAGCTGCTGCCGCCCGGCACTC

ratNPRC 301 GCTTCCAGGTGGCCTATGAAGACTCTGACTGTGGCAACCGTGCGCTCTTC

ratNPRC 351 AGTTTGGTGGACCGCGTGGCGGCGGCGCGCGGCGCCAAGCCGGACCTCAT

ratNPRC 401 CCTGGGTCCGGTGTGCGAGTACGCTGCGGCGCCGGTGGCCCGGCTGGCGT

ratNPRC 451 CTCACTGGGACCTGCCGATGCTGTCCGCAGGAGCGCTGGCCGCGGGTTTC

ratNPRC 501 CAGCACAAGGACACGGAGTACTCGCACCTCACGCGCGTGGCGCCTGCCTA

ratNPRC 551 CGCCAAGATGGGAGAGATGATGCTCGCTCTGTTCCGCCACCACCACTGGA
mNPRC     1 ..A.....K.M..G.GAGATGATGCTCGCTCTGTTTCGCCACCACCACTGGA

ratNPRC 601 GCCGCGCTGCCGTGCTCTACAGCGACGACAAACTCGAGAGAAACTGCTAC
mNPRC    38 GCCGTGCAGCCCTGGTCTACAGCGACGACAAACTCGAGAGGAACTGTTAT

ratNPRC 651 TTCACTCTCGAGGGGGTCCACGAGGTTTTCCAGGAGGAGGGCTTGCACAC
mNPRC    88 TTCACCCTCGAGGGGGTCCACGAGGTTTTTCAGGAGGAGGGGTTGCACAC

ratNPRC 701 GTCTGCCTACAATTTCGACGAGACCAAAGACTTGGACCTGGACGACATAG
mNPRC   138 GTCTGCCTACAATTTCGACGAGACCAAAGACTTGGACCTGGACGACATAG

ratNPRC 751 TGCGCTACATCCAGGGCAGTGAGCGAGTGGTGATCATGTGTGCCAGTGGT
mNPRC   188 TGCGCTACATCCAAGGCAGCGAGCGAGTGGTGATCATGTGTGCCAGTGGT

ratNPRC 801 GACACCATTCGGAGAATCATGTTGGCGGTGCACAGACACGGCATGACCAG
mNPRC   238 GACACCATTCGGAGAATCATGTTGGCGGTGCACAGACACGGCATGACCAG

ratNPRC 851 TGGAGACTATGCTTTCTTCAACATTGAACTCTTCAACAGTTCTTCTTACG
mNPRC   288 TGGAGACTACGCTTTCTTCAACATTGAACTCTTCAACAGTTCTTCCTACG

ratNPRC 901 GAGATGGCTCGTGGAAGAGAGGGGACAAACACGACTTTGAAGCTAAGCAA
mNPRC   338 GAGATGGCTCGTGGAGGAGAGGAGACAAACACGACTCTGAAGCTAAACAA

ratNPRC 951 GCTTACTCATCCCTCCAAACAGTCACCCTGCTGAGGACCGCGAAGCCTGA
mNPRC   388 GCATACTCGTCCCTCCAAACAGTCACCCTACTGAGGACCGTGAAACCTGA

ratNPRC 1001 GTTTGAGAAGTTTTCCATGGAGGTGAAAAGTTCTGTTGAGAAACAAGGGC
mNPRC   438 GTTTGAGAAGTTTTCCATGGAGGTGAAAAGTTCTGTTGAGAAACAAGGGC

ratNPRC 1051 TCAATGAGGAGGATTACGTGAACATGTTCGTTGAAGGCTTCCATGATGCC
mNPRC   488 TCAATGAGGAGGATTACGTGAACATGTTTGTTGAAGGGTTCCATGACGCC

ratNPRC 1101 ATCCTCCTCTACGTTCTGGCTTTGCATGAAGTGCTCAGAGCTGGCTACAG
mNPRC   538 ATCCTCCTCTACGTTCTGGCTTTACATGAAGTACTCAGAGCTGGCTACAG
```

On 7.15 pm
~~off 7.49 pm~~
10 V/cm

Dye	1.5% Agarose HT1
W	
A (undiluted)	1L 1R
B "	1L 1MR
C "	1ML 1R
W	
Acryl. marker	
W	
Dye	

Conclusion: The mouse pair, C, worked.

The rat sequence L1, is no good
Purify the 210bp, 3' end of exon 1, probe
& try Southerns etc.

Thurs. Aug 8th

ANP -CR probes

PCR formulation from A'121 for a 25 µl RX

checked with Kim

		ddw	n × 16.15
	A'121	20X PCR	n × 1.25 µl
	B'89	4dNTP's 25mM	n × 0.1 µl
	B'89	DMSO	n × 2.5 µl
14 µl		Left Primer	n × 1 µl
14 µl		Right Primer	n × 1 µl
	B'113	Standard Taq	n × 2 µl
	B113 E14 T6-22a	DNA 0.5 µg/µl	n × 1 µl
			n × 25 µl

New from Kim (8/4/94)

- or as suitable 500 ng is max. Can use 100 ng

Can add later

226 µl	
17.5 µl	
1.4 µl	
35 µl	
0	
0	
28 µl	
14 µl	
14×23 = 322 µl RX B'113 MIX	

Use n = 14

∴ expect to set up 100 µl RX for each primer set

Set up without primers.
Dispense 92 µl to each of :-

	1L 1R B'113	1L 1MR B'113	1ML 1R B'113
B'113 RxMix	92 µl	92 µl	92 µl
Primer 1L	1L 4 µl	1L 4 µl	
Primer 1R	1R 4 µl		1R 4 µl
Primer 1MR		1MR 4 µl	
Primer 1ML			1ML 4 µl
Expected size	615 bp	432 bp	210 bp
Label	A	B	C

On 12:23 pm
35 cycles
Set up first at 93°
for ~12 min extra
Then 1 min 93°/92°
7 sec 40° during
7 min 60° min

6.44 pm inspected
was turned Temp 46°

Shut off at 5 pm

08286
ade: 8-4-94
'II: 23
: 52%

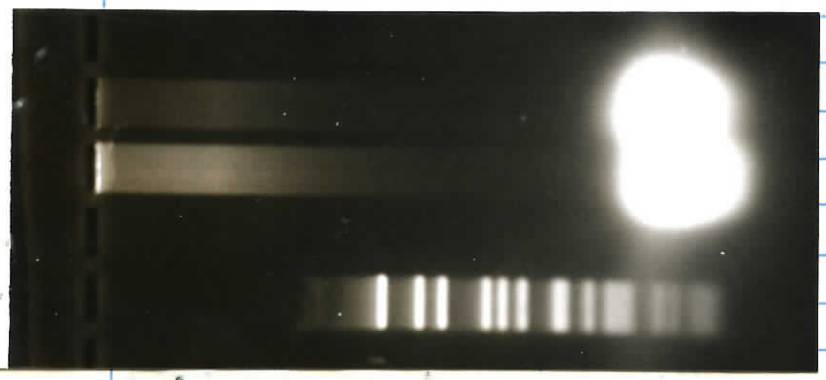
note: CR488-481 8 Right

5' TCT TCT CGC AGC TCT 3'
CGA TGC TT T
500 A 0.2 uM NAT-020-500 Prime Synthesis 004766

08287
ade: 8-4-94
'II: 24
: 50%

note: CR433-440 7 Left

5' GCC TAG AAG AAT CTG 3'
CAG TGA CAG
500 A 0.2 uM NAG-020-500 Prime Synthesis 005985



Dye
W
1ML 1R
7L 8R
W
Acryl. MW
W
Dye
1.5% HX1
on 10.27am
off 10.55am
10V/cm

Conclusion: Something wrong! Looking back – no check mark on 20xPCR buffer! Post gel is not good for 200bp. Re-run on 2% OK was new

Primer volume wasn't!

Fri. Aug 5th

Intran 7 Clearance Receptor probe

- unknown length ∴ use 10 min

PCR formula:-

Used the B'113 recipe for 14 tubes

Divided into 2/3 & 1/3

207 µl

Rest (115 µl)

≡ 9 tubes
+ 36 µl 8R ✓
+ 36 µl 7h ✓
etc to 8 × 35 µl

Blue dot

≡ 5 tubes
+ 20 µl IR ✓
+ 20 µl IML ✓
To 4 × 38 µl
4 135 µl

Black dot

Started at ~ 12:20 pm

93° for ≥ 2 min 1st cycle 1 min rest
40° 7 sec
60° 10 min.

Note

Expected finish 6:50 pm - it did.

Discarded blue samples, kept black

Conclusion: 1ML1R probably worked, but gel is not suitable
Re run (2 from B'115) at 2%.
Put stained gel at 42° + stain overnight to look. (Confirms)

The Blue product at 600bp is conceivably correct.
Would need to amplify with nested primers to tell.

Check for prep. gel

	Dye	
	W	
1ML1R	Black	B'115
1ML1R	Black	B'117
	W	
	Marker	
	W	
	Dye	

Conclusion: 2% Seap. will seperate well
Don't know why B'115 is -ve.

Make new Seaplaque
The Seap. was filtered thro' a metal filter - must have been contaminated.

Put record time - but dye - not normal

1.5% Ag HX

2% Seap. HX

75% Max (~7.5V/cm)

Sat. Aug. 6th

Repeat of 7L–8R CR

n =	14			
✓	226		ddw	16.15
✓	12.5	A'121	20×PCR	1.25
✓	1.4	B'89	4dNTP's 25mM	0.1
✓	35	B'89	DMSO	2.5
				Left primer 1
				Right primer 1
✓	28	B'117 (B'115)	Standard Taq	2
✓	14	B'113	DNA 0.5μg/μl	1
	322			23

214μl (≅ 9.3 tubes)

✓ 9.3μl 8R

✓ 9.3μl 7L

107 (Rest) μl (≅ 4.7 tubes)

✓ 4.7μl 1R

✓ 4.7μl 1ML

Dye
Water
Black 1ML 1R
Blue 7L 8R
Water
Ac. MM
W
Dye

To 4×55μl To 2×55μl tubes

1.5% HX1
Max 10V/cm
On 7.42pm
Off 8.12pm

~1.05pm Started with ≥1min extra melt + normal 1min @ 93.5

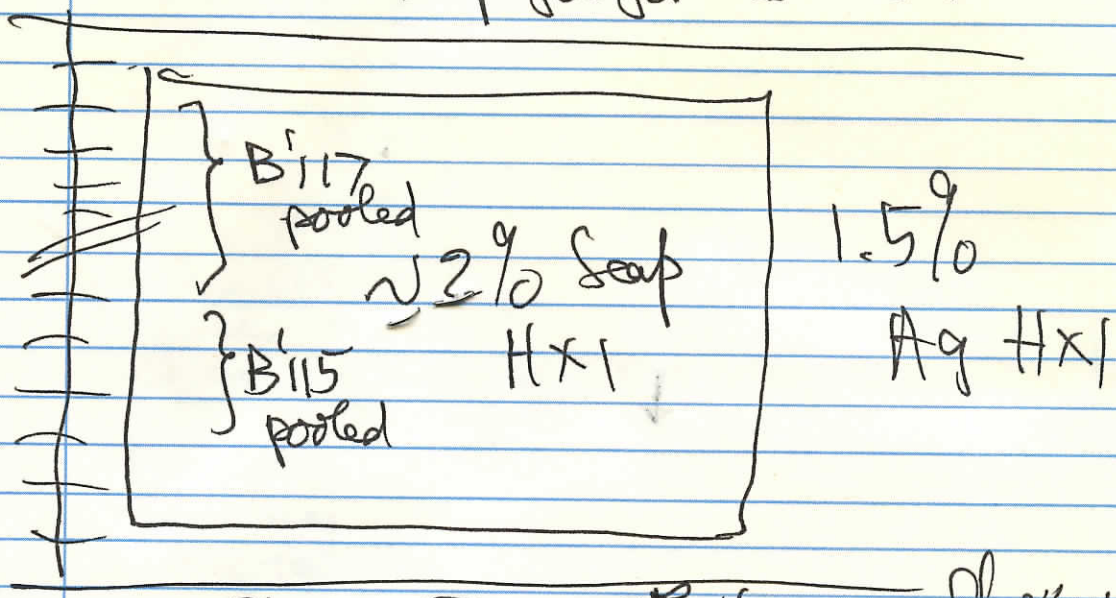
Program 1 min 93
7 sec 40°
10:00 min 60°

Sh'd finish ~ 7:35pm ✓

Closer to 59.5°C

Mon Aug 8th

Prep. gel for 1ML1R



On 3:54 pm 75 V/cm Off ~4:40 pm

Only the B'117 had any band – but B'115 was a recovered sample anyway.

Stained. Cut out. Sylvie will recover at

B'117
210 bp
Exon 1

✓ It worked well.

4-34 marker

Mon. Aug 22nd

Two hypertension papers off, back from the West Coast adventure, shingles 98% finished!

5' Clearance Receptor probe

S.H. tested B'117 fragment on Southerns – reasonable with 0.1×SSC. Paul had better try to improve the chances. Work on rat L1 + mouse 1MR using different conditions &/or rat DNA.

Naomichi will test B'108 1 Left with 1 Midright with mouse &/or rat DNA using rather milf conditions: 94° 1 min, 50° 2 min, 65° 5 min + DMSO } B'121 program

ON 11.25am 1.5% H 10V/cm

~~Off~~

~~Dye~~
Water
21 Mouse DNA 1L 1MR from Naomichi
22 Rat DNA . . . . . .
10 7L 8R Mouse DNA . . . . . .
Water
~~AC MM~~
~~Water AGMM~~
~~Dye~~

Conclusion: 7L/8R works well, as known. 1L/1MR may be okay – see arrow – expected size is 434. Redesign nested inner primers & try again.

le: _____

I: 20
65%

5' note: 1L2 3'

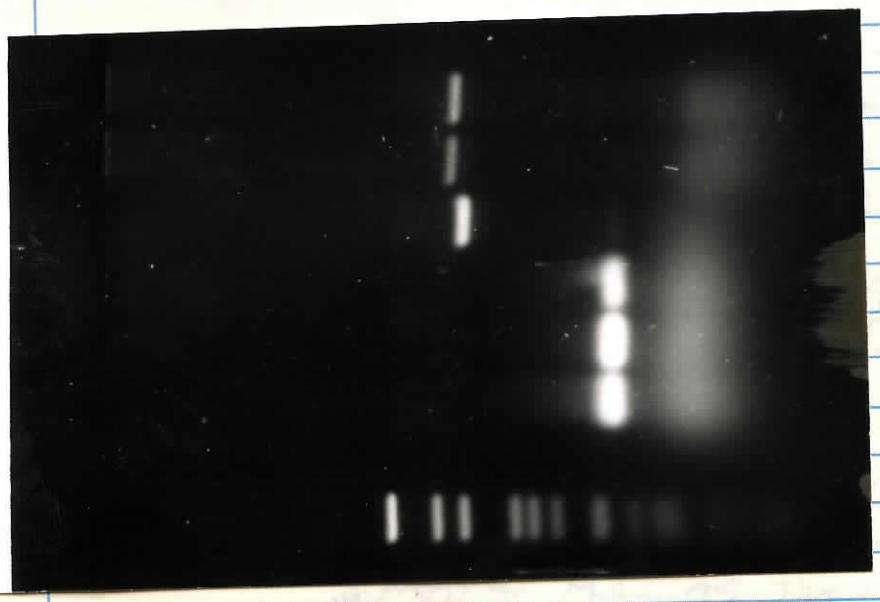
C	T	A	T	T	G	C	C	C	C	G	G	G	A	C
G	A	T	T	C										

le: _____

I: 20
65%

5' note: 1MR2 3'

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



0.01 µl
0.1 µl
1 µl

Tues. Aug 23rd

1L2 and 1MR2

Nested primers for 1L and 1MR.

Expected 383bp from the 434bp using 1L and 1MR

Naomichi ran with B'121 program & got final amplification - size ↓ slightly - with 0.01µl, 0.1µl and 1µl of 1st round 1L/1MR amplification.

S.H. will make more & test.

Sequence: 08480

Total bases = 20
A= 3, G= 7, C= 6, T= 4, 5= 0, 6= 0, 7= 0, 8= 0
(mixed bases= 0)

MW: 6128.0

5'> CCC ATC TTG GCG TAG GCA GG <3'

1MR2

Sequence: 08479

Total bases = 20
A= 3, G= 5, C= 7, T= 5, 5= 0, 6= 0, 7= 0, 8= 0
(mixed bases= 0)

MW: 6065.0

5'> CTA TTG CCC CGG GAC GAT TC <3'

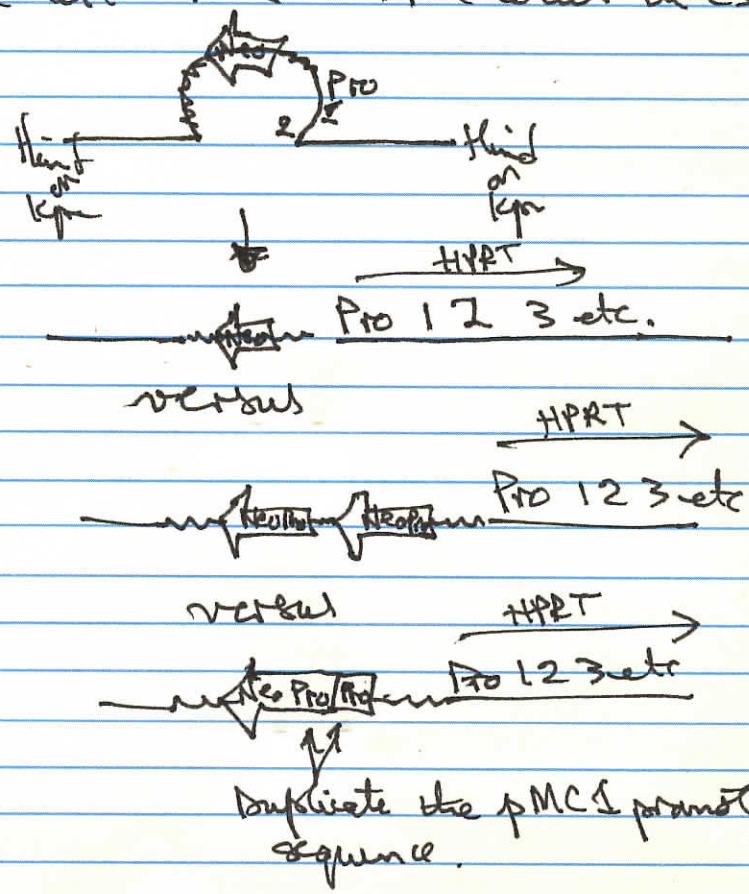
1L2

Mon. Aug. 29th

Promoter Duplication

Over the last few weeks I have been considering how to compensate for ↓ in β globin expression consequent to selective ↑ or advantage genes within the locus. One possibility is to duplicate the whole gene but cannot make a useful circle cell model this way, or to put in an LCR or to duplicate the promoter.

Easiest test of this is with Neo (known to show effects of gene duplication -- higher [G418] resistance). Can test in the HPRT locus in ES cells:-



also available as HindIII

pMCINeo'polA

XhoI

CASSETTE

Enhancer (1)

Hs at eu II

Taq I 2

TCGAGCAGTCTGGTTTTGCAAGAGGAAGCAAAAAGCCTCTCCACCCAGCCCTGGAATGTT
1 ---------- 60
AGCTCGTCACACCAAAACGTTCTCCTTCGTTTTTCGGAGAGGTGGGTCCGGACCTTACAA

Enhancer (2)

Taq I 2

Hae I

TCCACCCAATGTCGAGCAGTGTGGTTTTGCAAGAGGAAGCAAAAAGCCTCTCCACCCAGG
61 ---------- 120
AGGTGGGTTACAGCTCGTCACACCAAAACGTTCTCCTTCGTTTTTCGGAGAGGTGGGTCC

Sty I

Taq I I 2

Taq I I 2

SV TK Promoter

Eco RI

CCTGGAATGTTTCCACCCAATGTCGAGCAAACCCCGCCCAGCGTCTTGTCATTGGCGAAT
121 ---------- 180
GGACCTTACAAAGGTGGGTTACAGCTCGTTTGGGGCGGGTCGCAGAACAGTAACCGCTTA

Red C See F'101

Nsp V

Msl I

TCGAACACGCAGATGCAGTCGGGGCGGCGCGGTCCCAGGTCCACTTCGCATATTAAGGTG
181 ---------- 240
AGCTTGTGCGTCTACGTCAGCCCCGCCGCGCCAGGGTCCAGGTGAAGCGTATAATTCCAC

Afl M II Iu II

Hae I

Sfc I

Pst I

Met

Gdi I I

Eae I

Neo

3-end

Bam

Sal

Hind

ACGCGTGTGGCCTCGAACACCGAGCGACCCTGCAGCCAATATGGGATCGGCCATTGAACA
241 ---------- 300
TGCGCACACCGGAGCTTGTGGCTCGCTGGGACGTCGGTTATACCCTAGCCGGTAACTTGT

Transl^n initiation sequence

Taq

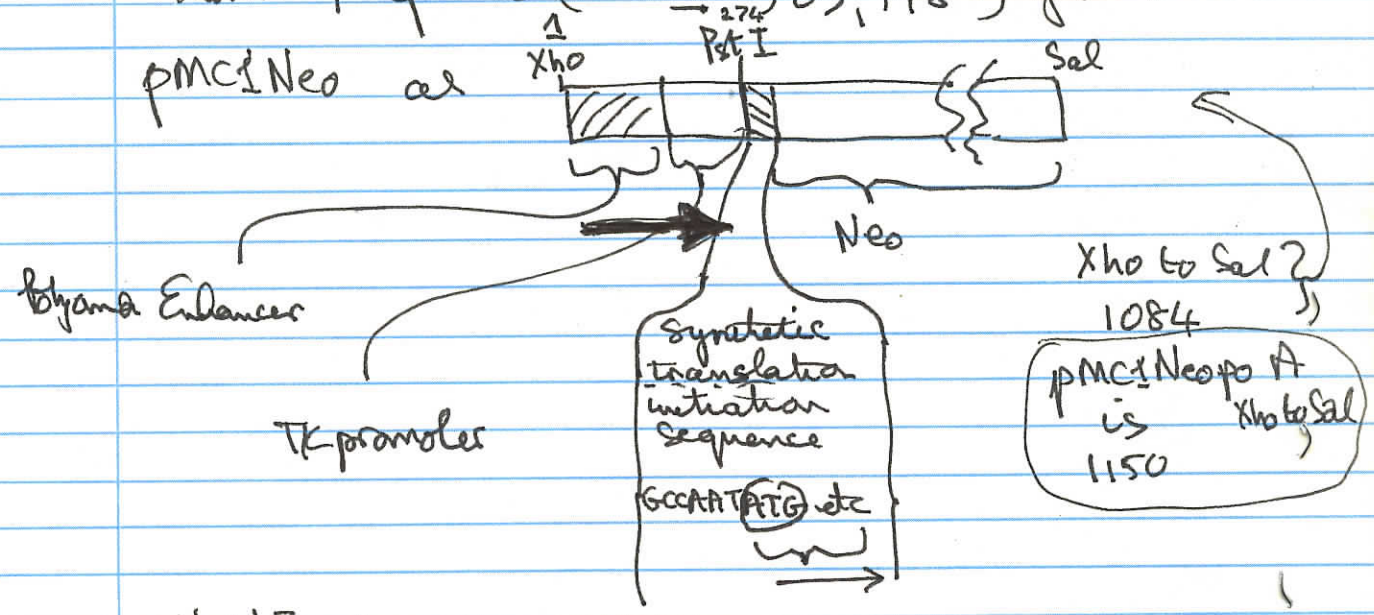
B

Gc

Thurs. Sep 1d

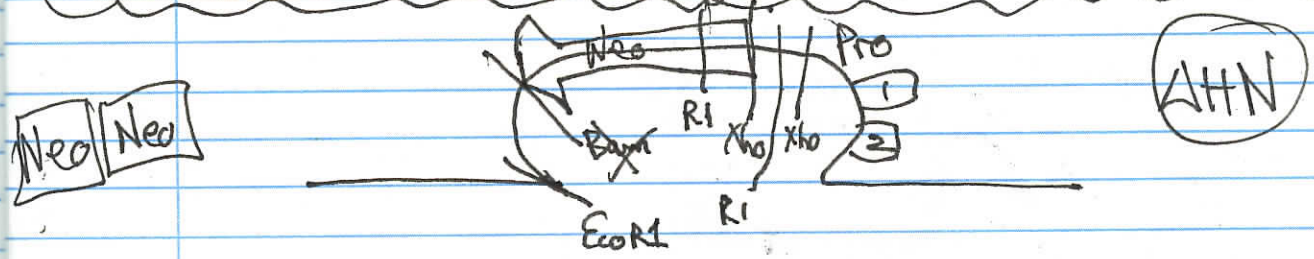
Promoter & Neo Duplication

Thomas & Capecchi (Cell 51, 503, 1987) give pMC1Neo as



Hind 3 or Xho I — Pst I is the piece to duplicate 274 bp for [Pro][Pro][Neo]

Xho I — Sal I or Hind I or BamHI or from pMC1Neopo A is the one for [Neo][Neo]



Cut with Xho I & insert Sal I ⟨└┘⟩ Xho I into site. Check orientation (Sal goes into Xho) by Xho cut plus Bam.

G TCGA C TCGA

Oligo # ~~2507~~ 5' note: OS PST→Xho

Date made: 9-1-94 C C T C G A G T G

LENGTH: 11

% G+C: 64%

NAA-020-500 Prime Synthesis 500 Å 0.2 uM

MW: 3315.2

5'> CCT CGA GTG CA <3'

Backup into 0.5ml TE 1XCIAA Assume 1µg/µl

CCTCGAGGTGCA

O.S. Pst→Xho B'128

Uncut
Sal
Xho
Sal/Xho
Pst
Pst/Xho

on 5:41 pm
~~off 6:41 pm~~

0.8%
1x4
max

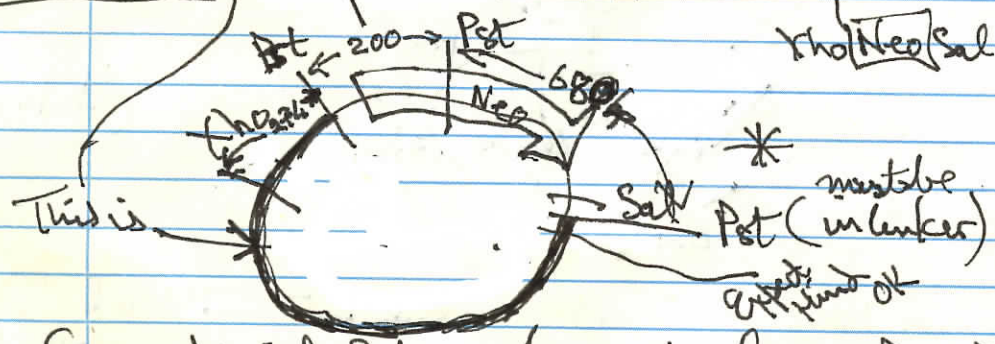
Dye
W
~~Uncut~~
129 Xho
129 Sal
129 Sal Xho
129 Pst
129 Pst Xho
W
Marker
W
Dye

pMCI

Xho Pst (desired) E-Pro

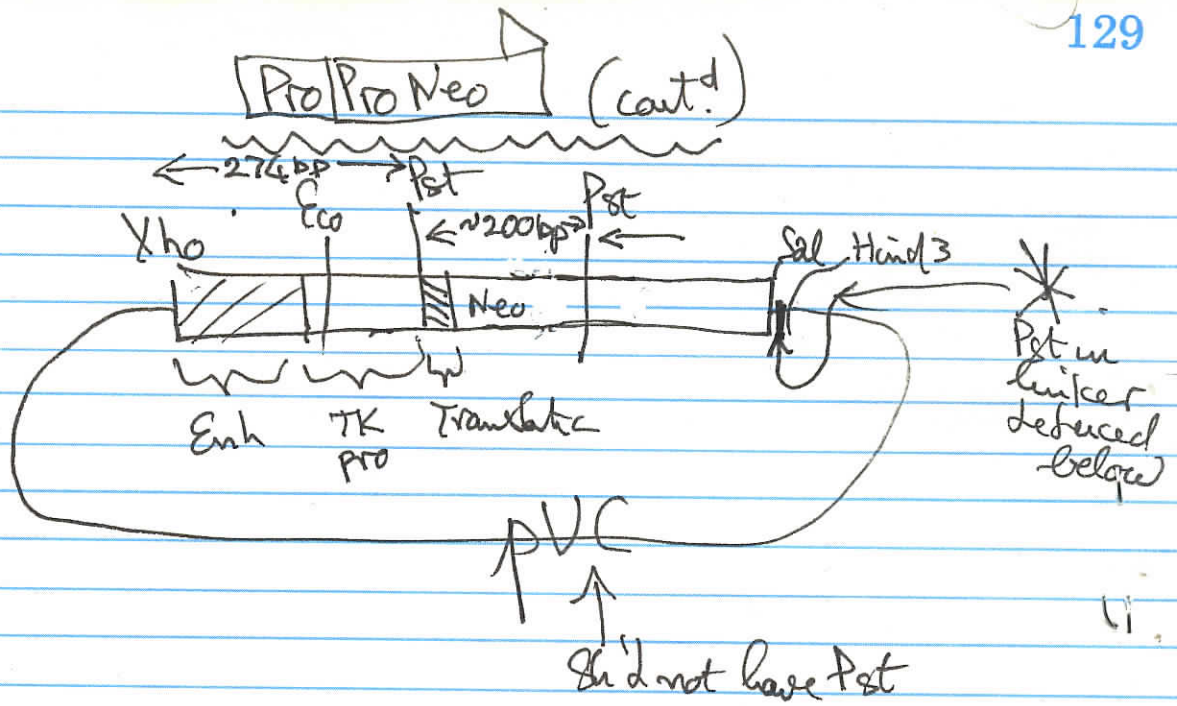
Conclusion: Map must be:—

Xho Neo Sal



So cut with Pst, as planned, change Pst→Xho with linker & go!

Check ΔHN for one (effectively) Xho site



Cut with Pst; expect to ↓ length by ~200 bp
– check against Xho cut. Change Pst to Xho.
pMC1 Neo — Excise Xho — Xho & put into AHN

∴ take 37 µl (1.5 µg) ψ30 ~~From NM (now labelled D'128)~~ 6877 ~~pMC1 Neo pol A~~ really SHI 114 0.46 µg/µl

✓ 14 µl 10x R1 salts
✓ 123 µl ddw

Transferred to D'153
~~Moved to~~ "Miscelaneous"

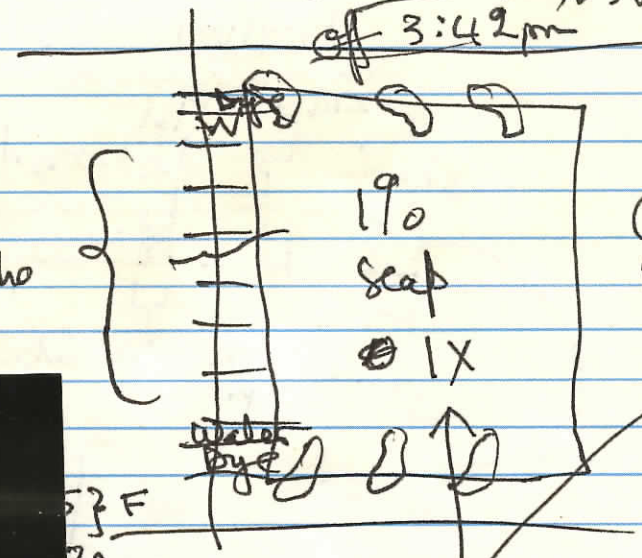
Rest ✓ 20 µl ✓ 20 µl ✓ 20 µl ✓ 20 µl ✓ 20 µl

Uncut
+ ~0.2 µl Xho I ✓ Lot 27 20u/µl
0.5 + µl Sal I Lot
Lot 31 10u/µl
~0.2 0.2 µl Xho I ✓ Lot 27
+ ½ µl Sal I ✓ 0.5µ
+ 0.3 µl Pst I Lot 35 20u/µl ~0.2µl
+ 0.2 µl Pst I ✓ ~0.2µl
+ 0.2 µl Xho I ✓ ~0.2µl

129 X | 129 S | 129 SX | 129 P | 129 PX

37° 2:35pm → 5:25pm

on 2:41 pm ~ 250 mA
~ 5 V/cm (total)
off 3:42 pm



B131 Neo
Sal/Xho
~40 µl
1 µl

} F
} M
} S

Error but proceed with Neo/Sal/Xho

+ 80 µl 5M NH_4Ac
melted + + ΦOH/HOQ
Then 1X CIAP. Stored as [B130 F] [B130 M] [B130 S]

0.06 ~0.09 µl

B131
KH21
Xho/CIAP
/CIAP

Whole of B131 KH21 Xho
+ 10 µl 1M Tris pH 8 (5X CIAP buffer)
+ 1 µl lab CIAP
30 min 55°C 3:37 pm 4:07 pm
1X HOQ/HOΦ
2X CIAP

Fri. Sep. 2nd

Neo/Neo & PreProNeo (cont'd)

Take 11 µl (5 µg) B'129 pMC1 Neo polA (moved to p'153 2 to 'miscellaneous')
5 µl 10x R1 salts (~0.1 µg/µl)
34 µl ddw

10 µl (1 µg)
+ ~0.5 µl Lot 39 Pst I 20u/µl

Rest (4 µg)
+ 1 µl Xho I Lot 27 (20u/µl)
+ 1 µl Sal I Lot 31 Dec (20u/µl)

B'131 Neo/Pst → For linker lig^n

B'131 Neo/Sal/Xho → For prep. gel of Neo

Take 2.2 µl (4 µg) KH21 φ118 2K — Pro H1 H2 — H (or vice versa) H ..... K (1.85 µg/µl)
4 µl Eco R1 salts (~0.1 µg/µl)
34 µl ddw

Rest + 1 µl Xho I

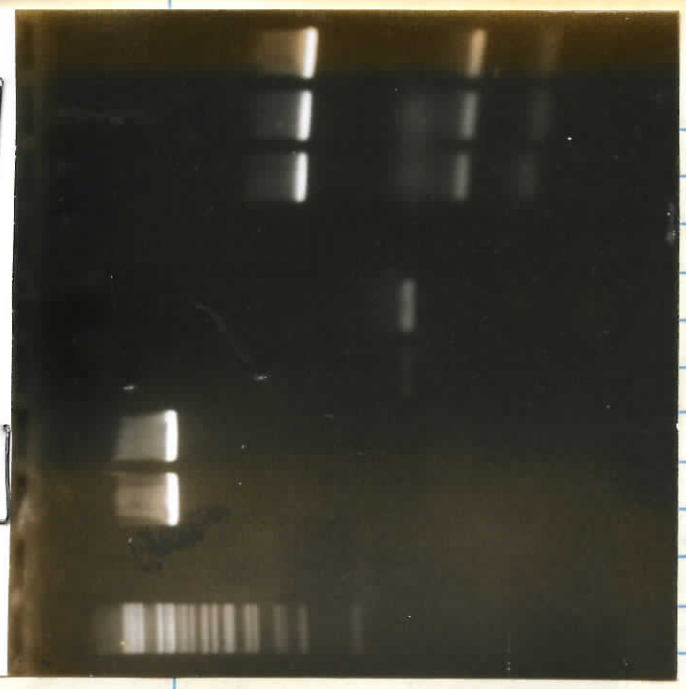
B'131 KH21 Xho → For CIAP

2.15 pm (except Pst)

2 µl (~0.2 µg)
+ 2 µl 10x R1 salts
+ 18 µl ddw
+ ½ µl Hind 3 Lot 46 (20u/µl)

B'131 KH21 Hind → For length control

2 µl + 2 µl POST Xho (37°, 11:24 am)



Conclusion:
linker conc^n appears very low - but both small bands got larger.
B'130 M is usable.
B'131 KH21 Xho has single cute.
Proceed.

On 11.57am
1: 12pm
8% HX4 MAX

	Dye
	W
+15	B'129 Pst
2+16	B'133 Pst
2+16	B'133 Pst
Undil^d	B'130 F
	B'130 M
	B'130 S
Undil^d	B'131 KH1
2+20	B'131 KH4
	W
	Marker
	W
	Dye

45
NdeV/a
b30

+15 pl
W5
linker
Linker

21 Hin
Xho

Che

Neo | Neo Pro | Pro Neo (cont'd)

Take whole (10µl) of B'131 Neo Pst
+ 2µl (1µg) B'128 OS Pst → Xho linker
+ 6µl ddw
+ 2µl p95 10x lig^n buffer (2/3/86)
+ 1µl T4 ligase

2µl + 16µl Pre B'133 linker lig^n

15° 1:14pm to 1:54pm
RT 1:54pm to 2:15pm
37° 2:15pm to 2:50pm — Rest to cold

2µl + 16µl Post B'133 linker lig^n

Transformation 10µl (~0.05µg)
80µl ddw
10µl 10xPBS
85° ~3min & to ice

(Don't need purif^n as the prep has ~zero ss DNA) → B'133 diluted

Transformation B'133 Neo Xho/Xho ≡ Neo ΔXX

B'133 DH5α 8/4	80µl
B'133 diluted	10µl (~0.05µg)
0°–3.12pm	~3.50pm
42°–3°	90sec

Plated Rest 20 5 20/16 5/16 37° 4pm
(220) (9) (3)

Check menis wrt Hind and Xho of B'128

Menis set up (–25 To 37° 12:40pm) B'133 Neo ΔXX

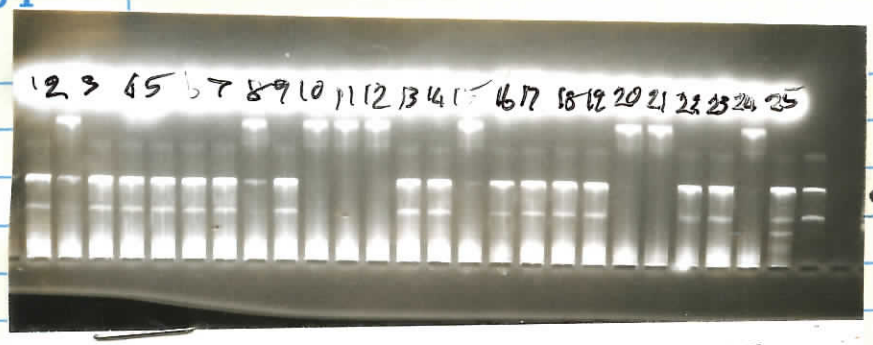
Only just about enough

Return to 1X per plate

Processed to 1X vol but use more for digests

Further too much 12:50pm

(1)

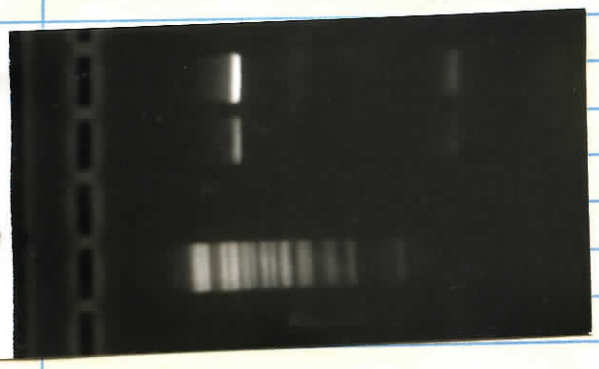


← KH21

Mar Sep 5th

Conclusion: Pick some more! Run pre/post lig.
If none – restart. If 1 or more, pick more.

(2)



Conclusion:
It was not a good lig.
Repeat with 200 µl B/30 M
overnight in cold
Check prior to trans.

(3)

Conclusion:
lig. was no good by all criteria
Take right B' 135²
repeated.

ON 9:32
~~off~~ 10:32
HX4 10x

(2)

Dye	
W	
Pre 135	Lig.
Post 135	Lig.
W	
Marker	
W	
Dye	

~0.2µl +1.6µl

0.8% H×4
on 5:30pm max 750mA
off 6:25pm

on 11.07am ditto
off 12:29pm

Sat. Sep. 3rd

1	
Dye	Dye
1	26
2	
3	
4	
5	
6	
7	
8	
9	
10	
11	
12	
13	
14	
15	
16	
17	
18	
19	
20	
21	
22	
23	
24	
25	
KH21	18
W	
Ag Marker	
Dye	

1–25 mind
B'135
20 Atc

50 undigested
Plenty left.

Neo | Neo

3

Take 8µl B'131 KH21 Xho/CIAP/CIAA
+ 100µl B'130 M
+ 12µl 8M NH_4Ac
+ 360µl EtOH
Dry Ice. Cent'd. 15µl
Back up into p95 x liq. buffer + 9µl dw 2µl 10X

B'135 KH21 Neo liq
+ 1µl T4 ligase

3 + 16 Pre 135

+1.5µl T4 to 4° at 2.05pm

4+14 Pre 135²

4+14 Post 135²

9.10am Wed (Sample) Tue

New tube B'135

3+16 Post 135

15° 5.14pm to 5.44pm
RT 5.44 to 6.12pm
37° 6.12pm to 6.42pm

B'135² KH21 Neo liq
8
200
21
690
18µl ddw

Transformation

DH5α 8/4 B'133	80µl
B'135 liq.	4µl
0° 6.45pm	to 7.15pm
90sec	42°
Plate Rest 20	5 20/16 5/16

B'135 KH21 Neo Neo

37° 7:25pm
11.30am

Mind 1 – 25 B'135 picked
To 37° 11.50am

26 – 50 (~2/3 x Amp)
10am Mon!

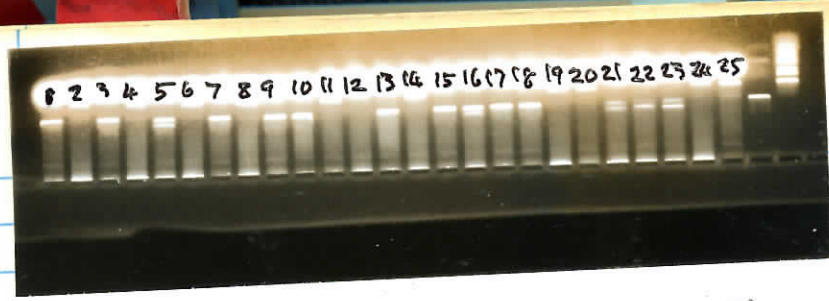
To 37° 7:30pm
Ma

Pre 135 Liq
Post 135 liq
W
Marker
W
Dye

135²

N 9.32am
f 10:32
H×4 08 max
Dye
W

N 0.2µl
+16µl



?

Conclusion: Initial look suggests 2, 4, 6, 11 (12) 14, 19 20, 24, 25 are OK. — w'd be ~ 40% i.e. Sfi + Pst

1, 3, 7, 9, 10, 13, 15, 16, 17, 18, 21-23 (?) prob. Sfi + Pst

Photo. again after RNA'se. Meanwhile digest 11, 14 & 12 with Hind III & with Xho I

2 4 6 8 11 ↑ 14 1920 2425 still look good.

12

Conclusion:
11 & 14 are okay. Can even use them to make the Xho Enh Pro Xho fragment using 1% Seap.

#14 streaked out as B137 pMC1 ΔNeo

On 11.05 am
Off 12.07 pm
0.8% 1X4 MAX

Dye
W
11 Hind
11 Xho
14 Hind
14 Xho
12 Hind
12 Xho
W
Marker
W
Dye

On 5:05pm 750mA
Off 5.50pm
0.8% H X4

Pst → Xho

Sun. Sep 4th

pMC1Neo Pst → Xho

Take
5 µl (2×28) µl 10× Eco R1
441 µl ddw
7 µl (0.25×28) (5u/sample) lot 35 Pst I (20 u/µl)

25 × 18 µl for 9 µl of each mini B'133 1–25

32° at 12:32pm
4.32pm

B'137
P1–25

Dye
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25

B'137
Pst
1–25
mini.

15 µl	B'133 diluted
	Acr Marker
	W
	Dye

6:30pm
gel to ~55°
with RNA'se.
200µg + Tris buffer
in ~400ml

B'137
RNA'se
6/12/94
lab stock
10µg / µl

Take ✓ 2×8 = 16 µl 10× Eco R1
✓ 18×8 = 128 µl ddw
144

✓ 70 µl
+ 1 µl Xho I lot 27
18 µl

✓ Rest
+ 1 µl Hind lot
18 µl

+2 µl mini [11 X] [14 X] [12 X]

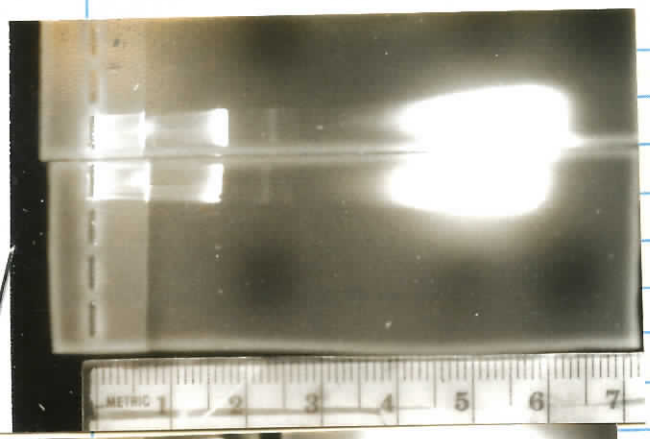
+2 µl mini [11 H] [14 H] [12 H]

37° 6.55pm to ~9pm

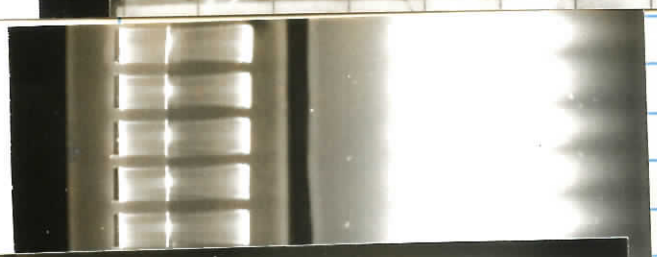
25
Pst – Pst

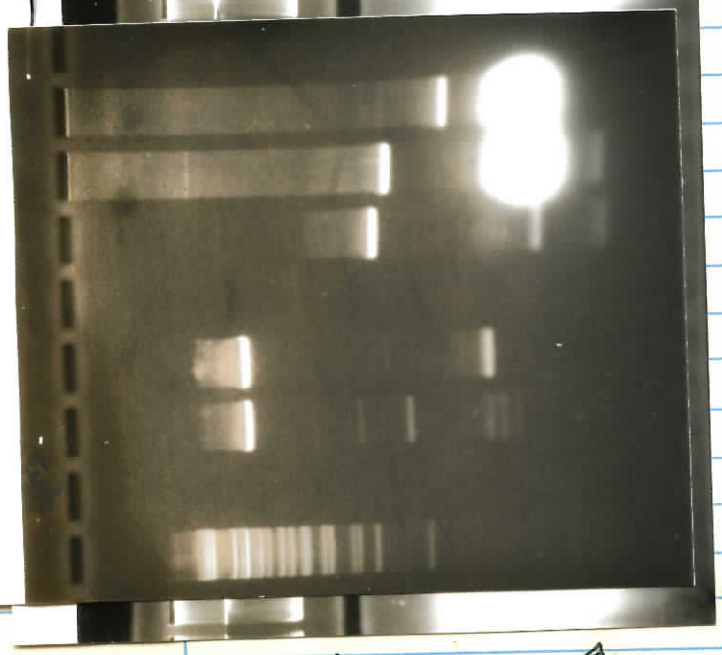
5am
07pm
0.8%
X4
max

Hind
Xho
Hind
Xho
Hind
Xho
L
Marker
W
Dye



Conclusion:
Difficult to believe — I'd have run with B'133 dil'd control.
Cut out a 2.2 g band anyway at ~2.3–2.8 cm — frozen.
Prob'ly but ~50%.
Prepare a test — later.





Conclusion: The digests of pooled minis worked fine. Prep gel first not. Use 0.8% Ag HX4 & cut for electroelution.

The B'135² lig'n worked well — but (as expected) a lot of circularization of insert. If unsuccessful, repeat at higher conc'n. Meanwhile transform & screen. Also ↓ amount of KH21.

Same mixed

Reversed

Tues. Sep 6th

Prep. gel for Xho [Enh. Pro] Xho

Take 40 µl 10 x R1 salts
✓ 280 µl ddw
✓ 40 [redacted] µl of B'133 mini #25
✓ 40 µl of B'133 mini # [redacted] 11
400µl
✓ +8µl (160u) XhoI Lot 27
37° 3:40 pm to 5 pm

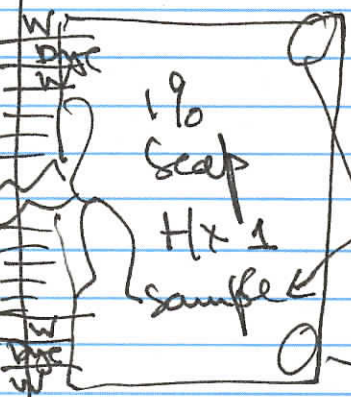
✓✓ 8+8 Pre [redacted] Xho

B'139 Xho

✓✓ 8+8 Post Xho

On 10:15 am
Off 11.15 am
0.8% HX4 Max

	Dye
	W
8+8	B'139 Pre Xho
8+8	B'139 Post Xho
Undil'd	B'133 Diluted
	B'139 FRAG
4+14	B'135² PRE LIG^N
4+14	B'135² POST LIG^N
	W
	Mark
	W
	Dye



1.5 [redacted]% Ag HX1
On 5:20 pm ~6V/cm 350mA
Off 6.25 pm

Yellow

2.2g recovery
1x QOH/HOQ
1x CLAA
+7 ml EtOH
"Freeze". Cent 15K
in yellow top. Back into 100µl

~~B'139 XXFRAG~~ Not kept

July 12, 1992 18:52 ..

Also available as Hind 3

May need hot start to avoid this primer but only 9 or 10 Bp.

```
                                                                                  T
                                                                                  a
                                                                                  q
                                                                                  I
                                                                                  I
                                                                HS                -
                                                                at                2
                                                                eu
                                                                II
                                                                /
XhoI
Not Y  |  X
TCGAGCAGTGTGGTTTTGCAAGAGGAAGCAAAAAGCCTCTCCACCCAGGCCTGGAATGTT
1 ------------+------------+------------+------------+------------+------------+ 60
AGCTCGTCACACCAAAACGTTCTCCTTCGTTTTTCGGAGAGGTGGGTCCGGACCTTACAA

                                                                                  H
                                                                                  a
                                                                                  e
                                                                                  I
TCCACCCAATGTCGAGCAGTGTGGTTTTGCAAGAGGAAGCAAAAAGCCTCTCCACCCAGG
61 ------------+------------+------------+------------+------------+------------+ 120
AGGTGGGTTACAGCTCGTCACACCAAAACGTTCTCCTTCGTTTTTCGGAGAGGTGGGTCC

                                                                                  E
                                                                                  c
                                                                                  o
                                                                                  R
                                                                                  I
                                                                                  F
                                                                                  s
                                                                                  t
                                                                                  I
S                                                                                 /
t                                                                                 /
u
I
/
CCTGGAATGTTTCCACCCAATGTCGAGCAAACCCCGCCCAGCGTCTTGTCATTGGCGAAT
121 ------------+------------+------------+------------+------------+------------+ 180
GGACCTTACAAAGGTGGGTTACAGCTCGTTTGGGGCGGGTCGCAGAACAGTAACCGCTTA

N
s
p
V
TCGAACACGCAGATGCAGTCGGGGCGGCGCGGTCCCAGGTCCACTTCGCATATTAAGGTG
181 ------------+------------+------------+------------+------------+------------+ 240
AGCTTGTGCGTCTACGTCAGCCCCGCCGCGCCAGGGTCCAGGTGAAGCGTATAATTCCAC

Reverse

A
f
lM
II
Iu
II
/
                                                                G
                                                                d
                                                                I
                                                                I
                                                                E
                                                                a
                                                                e
                                                                I
                                                                Met
                                                                ->
                                                                P
                                                                s
                                                                t
                                                                I
                                                                S
                                                                f
                                                                c
                                                                I
                                                                H
                                                                a
                                                                e
                                                                I
ACGCGTGTGGCCTCGAACACCGAGCGACCCTGCAGCCAATATGGGATCGGCCATTGAACA
241 ------------+------------+------------+------------+------------+------------+ 300
TGCGCACACCGGAGCTTGTGGCTCGCTGGGACGTCGGTTATACCCTAGCCGGTAACTTGT
Pro
```

Enh ->

<- Enh ->

FORWARD

ENH 2

X

Y ->

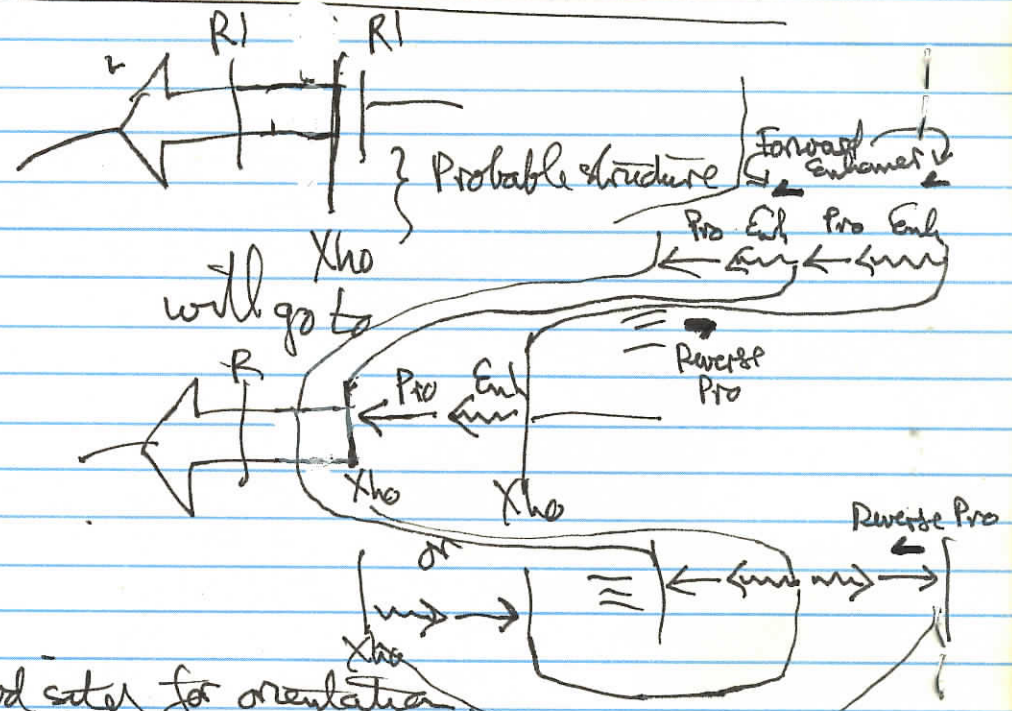
Y ->

3

Not X

Tues. Sep. 6th

Diagnosis of KH21Neo | Enh Pro |



No good sites for orientation.
∴ test with PCR:–

Inversion 1 primer will work Reverse Pro Forward enhancer

Can check on B'137 ↑MCI ↑Neo Pst { Direct Repeat Needs 2 primers Reverse Pro

B'141 FORENH | B'141 REVPRO

Back into 0.5ml TE/CIAA assume 1µg/µl

5' Oligo Name: FORWARD ENH B'141 3'

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

MW: 6667.4

8568 5'> TTT CCA CCC AAT GTC GAG CAG T <3'

5' Oligo Name: REVERSEPRO B'141 (G T C) 3'

			G	C	C		A															
	T	T	A		A																	

5'> GCC ACA CGC GTC ACC TTA ATA T <3'

	HAT	1X G418	1X G418 + HAT	1.7X G418	1.7X G418 + HAT	3X G418	3X G418 + HAT
KH21	15	1170	13	1230	24	1060	14
	14	1170	11	1070	19	820	14
NeoDup		1660	20	1210	18	650	20
		1400	14	1290	14	840	19
2PNeo		1390	17	1390	22	850	16
		1150	20	1050	18	740	17

SH U78 DNA's were :— KH21 $\phi 118^2$

NeoDup U75 #92

2PNeo SH U64 #78 (B'147 → #78 U47)

12/29/94

Conclusion: This assay detects no differences. ∴ picked 10-20 of HAT^R G418(1x)^R & some G418^R for use in assay of Neo activity.

Wed. Sep. 7th

Neo | Neo (repeat)

Transformation

B'143 DH5α 8/4	80 µl
B'135 2 ligⁿ	4 µl
0°C	12:15 pm to 1.08 pm
90 sec. 42°	2 min.
Plated	on (1x) Amp NZY (Rest) 20, 5, 20/16, 5/16
	on (2x) Amp 20

37° 1.20 pm to 10 am

Precautionary repeat ligⁿ :— Thurs. Sep 8th

✓ 2 µl (i.e. 25%) B'131 KH21 Xho/CIAP/CIAA
✓ 200 µl B'130 M
✓ 22 µl 8M NH_4Ac
✓ 672 µl EtOH
Dry Ice, Cent. Dry.

B'143 KH21 Neo ligⁿ

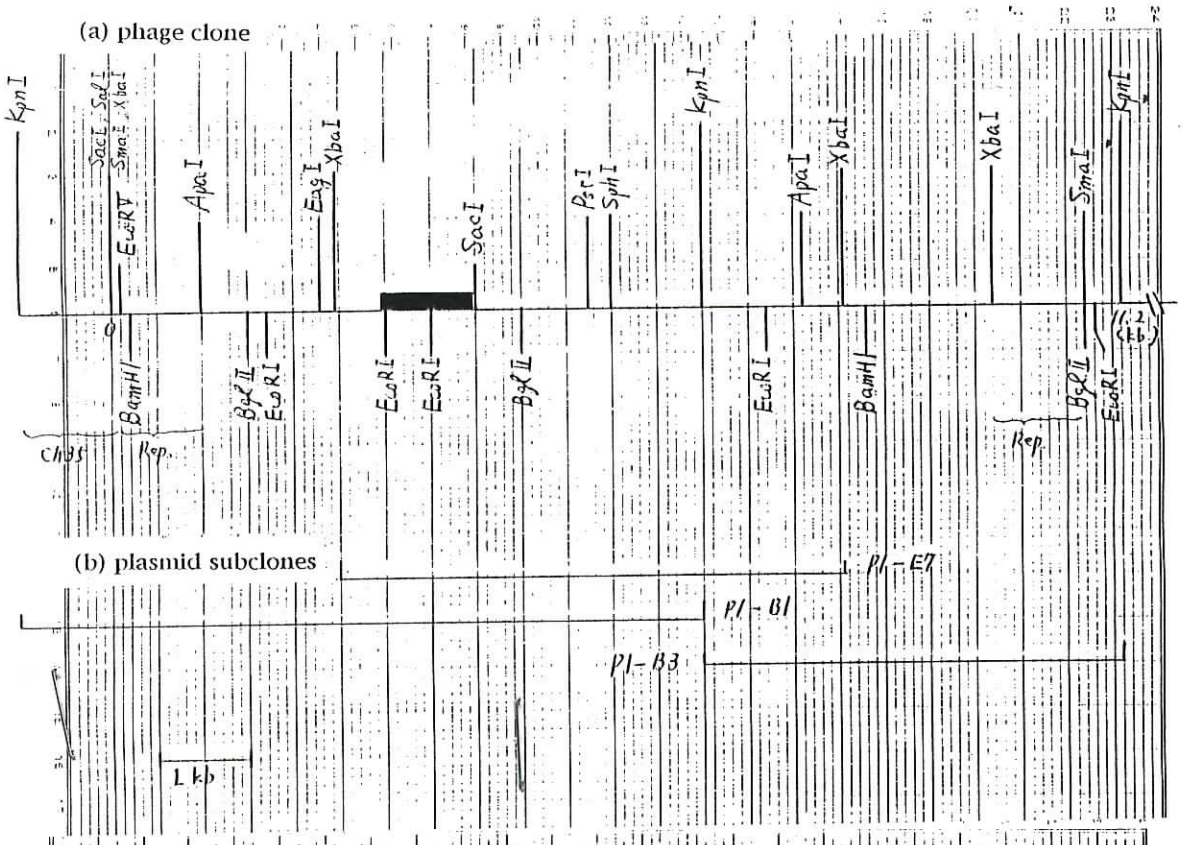
i.e 66% — Back into 10 µl 1x p95 ligⁿ buffer
1 µl T4 ligase B'135
Cold 9.35 am to 4 pm Fri

2+14 Ave 143 ligⁿ

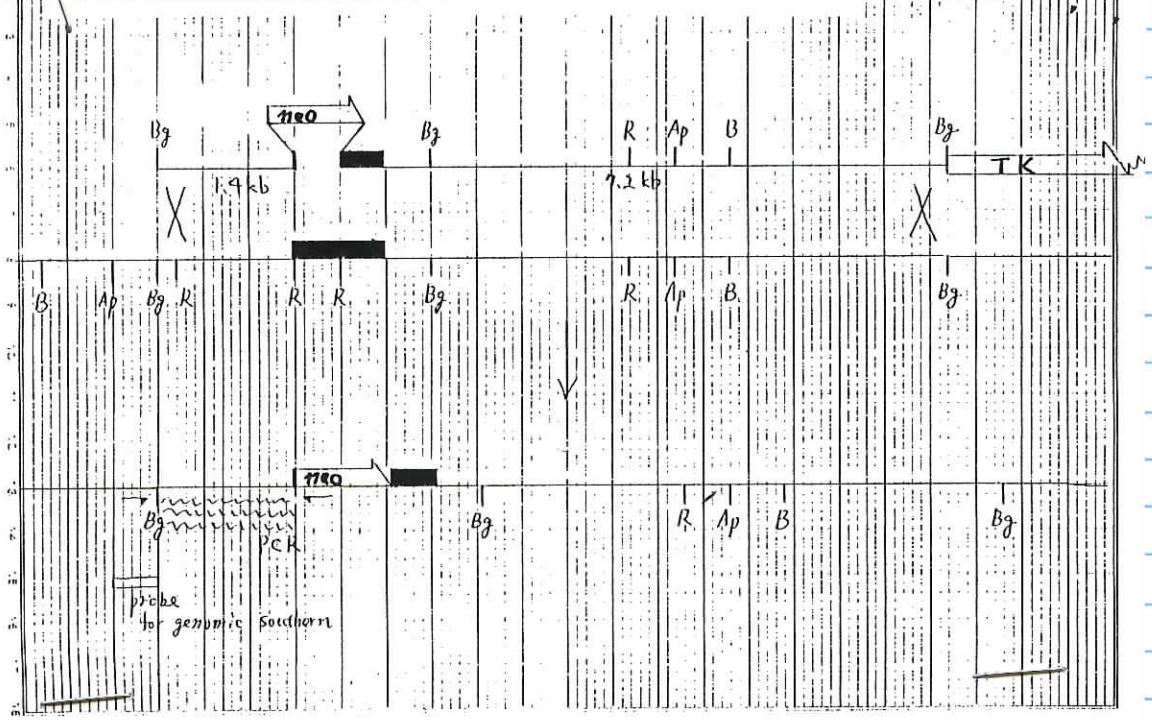
2+14 Post 143 ligⁿ

See SH U75 for Neo Neo KH21 product.

Restriction map of mouse angiotensinII receptor (Type 1A) gene



Targrted disruption of mouse angiotensin II receptor type 1A gene

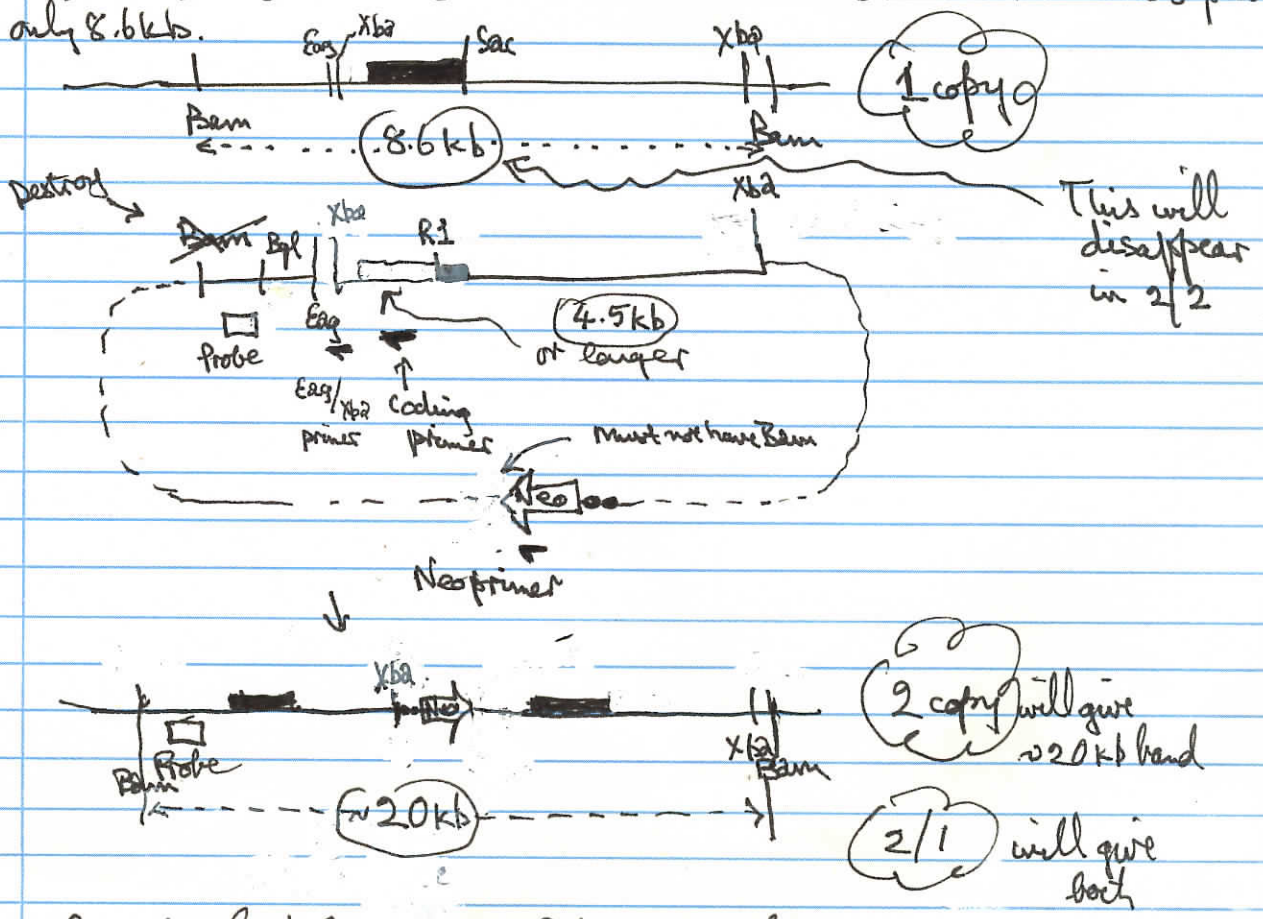


Tues - Nov. 15th Grants & papers v finished!

ATR1A Duplication

Masaki & Tom's success with ATR1A disruption & its effects on bp. (↓ Hg in +/− & lack of ATII responsiveness in −/− suggests that dup^n will ↑ bp. & test same concepts as ↑ NPR-CR clearance receptor (see B'107)

Can use the same 3' piece as Masaki — except for putting in a diagnostic site that will work with Southern for 2/1 versus 2/2. In fact no site within the duplication will work except quantitatively. Once again, either a 129 v. B6 difference is needed or duplication must be within scale of outside sites. Could use the Bam sites as Masaki did & duplicate only 8.6 kb.



Can also look for 129 v. B6 polymorphism.

(In)compatability of ends

Eag/Not — GGCC

Xba — CTAG

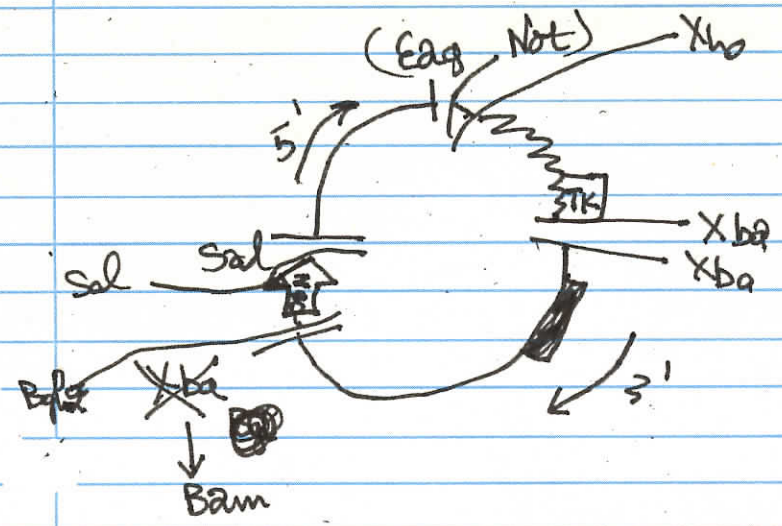
Bam/Bgl — GATC

Sal — TCGA

All four are discrete.

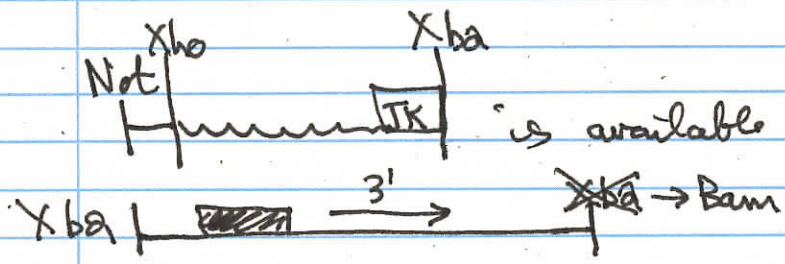
Wed. Nov. 16th

Initial plan for construct



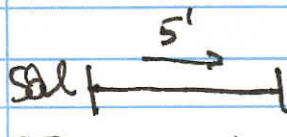
Linearize with Xho

Homology ~ 8 kb.

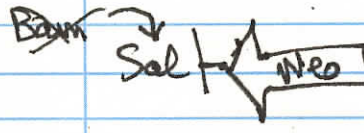


is available as B'45 TKS

can be made from P1-E7 with one site change



is directly isolatable from P1-B1



can be made from pMC1Neo with two site changes

← Primer for PCR detection can be made from within

Eag H Xba

→ Primer w'd be from 3' Neo.

PCR frag^t ~ 2.5-3 kb.

Comment The 5' duplicated gene will be very secure. The 3' copy may be somewhat short in promoter end.

5' Oligo Name: OSXBABAM 3

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

12
67%

5' Oligo Name: OS BAMSAL

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

Cancelled

5' Oligo Name: OS XHOBGL

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

14
57%

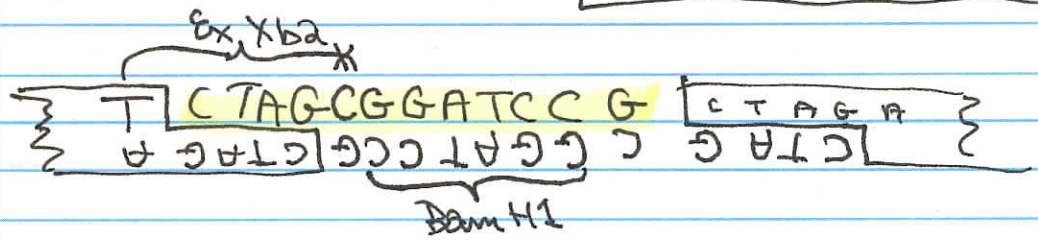
Into 0.5 ml TE
1 x CIAA
& labelled

Thur. Nov. 17th

Some adaptors

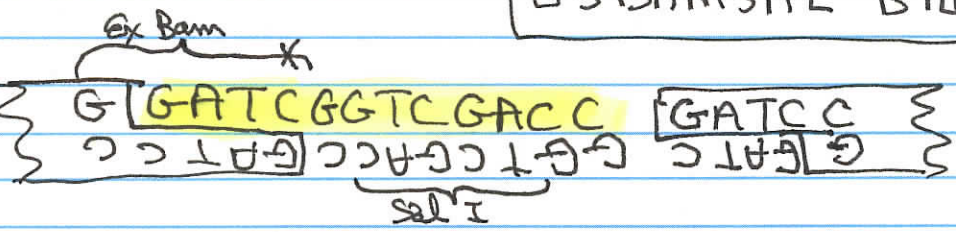
XbaI → BamHI

OSXBABAM B'149



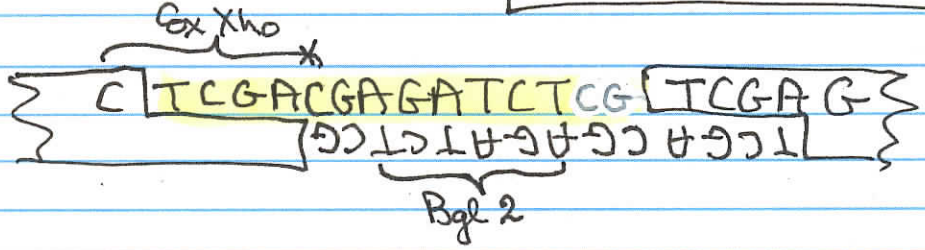
Bam → Sal

OSBAMSAL B'149



Xho → Bgl2

OSXHOBGL B'149



Transformation

✓ DH 5α 8/4 B'143	80μl
✓ B'151 LIG^N	20μl ≅ 0.1μg
✓ Ice 3.15pm	to 3:48pm
✓ 43°-42°	90sec
Plated	Rest 20 5 20/16 5/16

2X Amp

37° 4:02pm

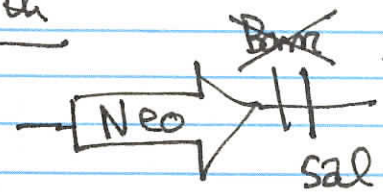
B'151
Neo ~~Bam~~

Picked 12 (with difficulty)
37° at 8.45am

N.M. already had made pMCINeo polA without Bam, so it cuts out with Xho/Sal

Thurs. Nov 17th

pMC1Neo ~~Bam~~



Can simplify process without linker by cut & fill.

Take 2.2 µl (1 µg) pMC1Neo (0.46 µg/µl) W87
2 µl 10x R1 Salts
14.8 µl ddw
½ µl Bam H1 (10 u) 20 u/µl lot 72

37° 11.08am → 11.50 pm [B'151 NeoBam] 0.05 µg/µl

1+20 [PRE B B'151]

1+20 [POST B B'151]

+ 1 µl 1mM mix of dNTP's [B'151 4 dNTPs 1mM each]
+ 1 µl Klenow polymerase ? u/µl lot 57

[B'151 Neo Bam Klenow] → 37° 11:55am → 12:57pm

Take 6 µl B'151 NeoBamKlenow
+ 6 µl p95 ligⁿ buffer
+ 46 µl ddw
+ 2 µl T4 ligase

[0.005 µg/µl B'151 LIGⁿ]

15 µl [B'151 PRE LIGⁿ]

37° 1:13pm to 3 pm

15 µl [B'151 POST LIGⁿ]

Gel → NW

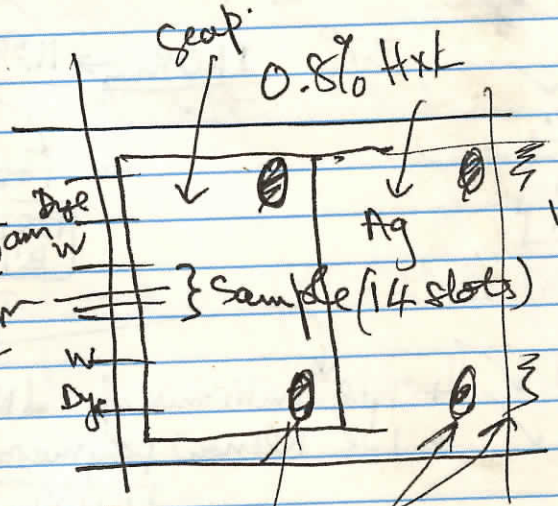
On 3:40pm
~~Off 4.55pm~~
0.8% Hx4 max

Dye
W
Pre
"0"
1
2
4
8
16
32
R
W
Marker
W
Post } Lig^n
Pre } B'153
Post } Bam
Pre } B'153
W
Dye

2+16
B'153
XbaI

15
15
1+20

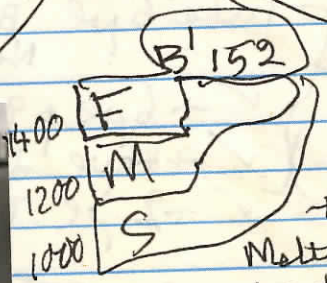
Conclusion:
Ligation & Bam were good.
Pool 0, 1, 2, 4, 8 & prepare single cut, or repeat in bulk at 2 min.



On 11:35am
~~Off 4:30pm~~
400mA
~3 V/cm

Sample is whole of B'153 PI-E7 X→B
+100µl ddW ✓

Dues



Weighed
+ 1/10th 8M NH_4Ac
Melt 65°
1x ϕOH/HOQ
2x CIAA

1x4 max

↓ to 2/3 at 4pm ↗ 1/x 4:30pm

Thurs. Nov 17th

Partial digests of P1-E7

0.05 pg/µl

B'153
XbaI

Taken ✓ 5 µl (~10 µg) P1-E7 ① 2.1 µg/µl 2/14/92
✓ + 20 µl 10xRS + 1.5M NaCl ← to help with heat inact
✓ + 175 µl ddw
✓ + 1/2 µl (10u) XbaI hot 20u/µl

Pre	0 (~1:55pm)	1 min	2 min	4 min	8 min	16 min	32 min	57 min
20 µl ✓	✓	✓	✓	✓	✓	✓	✓	Rest

To 85°–90° (80) for 5 min.

Each time point fil.d, after heating, 2 µl + 16 µl ~~for~~ ep.

Pooled 0, 1, 2, 4, 8 to total vol. of ~75 µl

B'153
X → B

✓ 75 µl Partial Xba P1-E7 (~5 µg)
✓ + 9 µl 10x p95 lig.n buffer
+ 5 µl B'148 OSXBA BAM
+ 2 µl T4 ligase 15° 2.55pm to 3.55pm

RT 2.40pm to 2.55pm 37° 3.55pm to 4.47pm
Frozen

Use now for prep. gel

Conclusion:
NM's material is as advertised!
Abandon B'150/151 expt. & proceed with B'155

On 2:15pm
~~Off 4:10~~

	Dye
	W
	Pre
1+15	Post X
	Post X
	Post X
	W
	Marker
	W
	Dye

+B
+S

B'154 #3 ~~plate~~ streaked out (see B'156)

B'154 B Neo S

80µl B'133	
1 Whole of B'155 Neo Bgl Heat	
0° 5.15 to 5.45pm	
42°	90sec
Plated	Rest 20, 5, 20/16, 5/16

37° at

#1–5 B'154

37° 10:20am

#1–5 3–5 3 v. small

#2 1 OK

#1 1

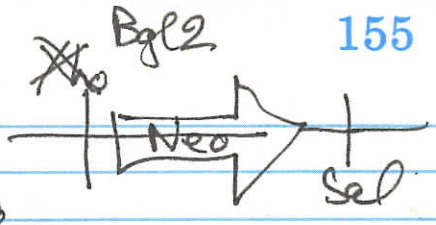
negative
A strain for Bgl2
37° 11am–11:40am

took B'155 X+B & added ½µl Bgl2

1+16 Pre B'154 Bgl2 | Post B'154 Bgl2

0.8% various voltages
HX4

Fri. Nov 18th



~~Xho~~ pMC1Neo ~~Bam~~
↓
Bgl2

B'155 +Bam +Sal

Take 4µl (~2µg) X135 #1 Midi (B'155)
(pMC1Neo pcA minus Bam from N.M)

+ 4µl 10x R1 salts
+ 32 µl ddw
20µl + ½µl (10u) Xho I Lot

10µl + ½µl Bam HI Lot

37° at 9.10am to 11.25am

B'155
Neo
Xho

1+15
Pre
B'155

10µl + ½µl (10u)
1+15 Sal I
Post B'155
X+S

1+15
Post
B'155
X+B

Take whole 19µl (~0.6µg) pMC1Neo (Bam+) Xho
+ 2.4µl 10x p95 high buffer
+ 1µl (~1µg) B'148 OS XHO BGL
+ 1 µl T4 Ligase
RT 2:40pm to 2.55pm

0.03µg/µl
B'155
Neo
→ Bgl2

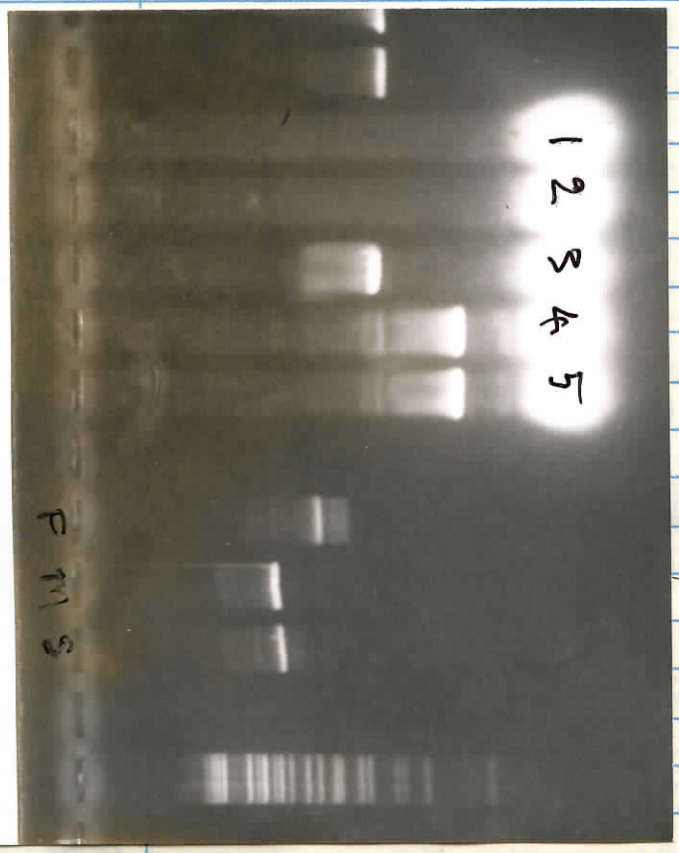
15° 2:55pm to 3:55pm 37° to 4.45pm

3.3µl + 2µl 10x PBS
0.1µg + 15µl ddw
85° for 3 min

B'155 Neo Bgl Heat

B'154

Conclusion: #3 mini has Pagl 2 – no site in original plasmid // Fragment M is good for ~~Xba~~ ——— Xba
↓
Bam H1



Sun. Nov 20 04

Minis etc.

Processed B'154 mini #1 – 5.

Take ✓ 12 µl 10x R1 salts
✓ 107 µl ddw
✓ 1 µl (200) Bgl 2 hot

B'157
B 1-5

1 µl of each mini to 20 µl 37° 12.15 pm to 2:53 pm
not fully dissolved

B'152 F 280 µl + 0.84 ml } 10% F into 20 µl
M 240 µl + 0.72 ml EtOH } 10% M
S 200 µl + 0.60 ml } 10% S !

on 3:50 pm
~~5:15 pm~~
0.8% H x 4 max

Dye
W
Pre Bgl 2 B'154 (Xho + Bam)
Post
1
2 } (B'154 Post Bgl 2)
3 mini
4 B'157 #1 – #5
5
Load B'152 prep gel
10% F
10% M
10% S
W
Marker
W
Dye

(1)

...s. processed minis as usual ~ 10am.
I digest is simplest initial screen for absence
...e XbaI site. Pam is better for +ve, but
...know if the vector has a site.

54 µl 10x RS [redacted] + 1.5M NaCl
+ 480 µl ddw
+ 6 µl (120 u) XbaI lot 29 (20 u/µl)
~ 4u?
... to 20 µl. Each +1 µl minis ← Pipetteman was a little erratic.

...59 #1 – #25 37° 11:35am to 2:15pm

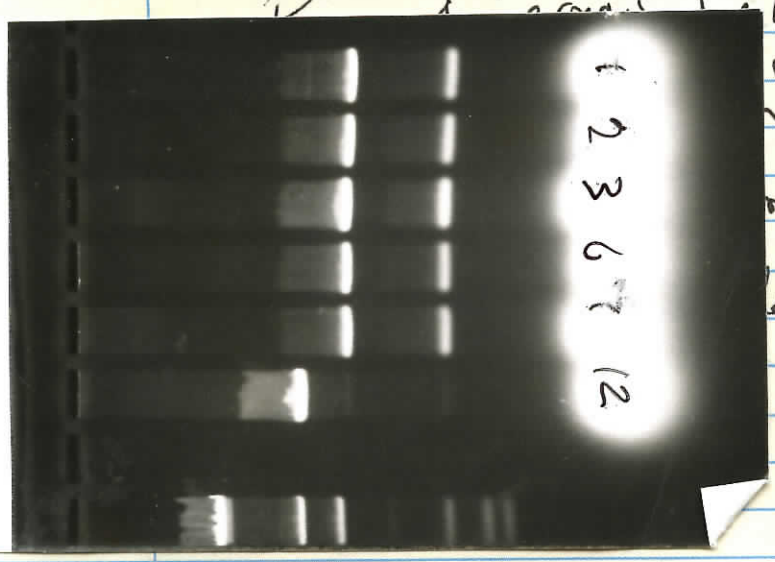
(1) Conclusion: 60% successful! Pick ~6 for orientation.
#'s 1, 2, 3, 6, 7 & 12
[redacted] will do fine.

Error – shi have used Sac I or Bgl 2

∴ take 14 µl 10x RS + 1.5M NaCl
+ 126 µl ddw
+ 1 µl (20 u) lot 29 XbaI
+ 1 µl (20 u) lot 43 Sal I
B'159
minis 1, 2, 3, 6, 7 & 12

0.8% HT4 MAX 6.45pm
off ~7.40pm

(2)



...later

See B'162 for finals

(2) Conclusion: #3 and #12 can go on to full test.

(2)
Dye
Water
1
2
3
6
7
12
W
M
W
Dye

B'159 XbaI Sal I minis

On 3pm
Off 4.05pm
0.8% HX4 MAX

Sun. Nov 20th

Eather → Bam or → Bam

Dye (1)
W
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
W
Marker
Dye

B'159 XbaI mini #1-#25

~10µl (equal to gel load) of B'157 "10% M" available
i.e. ≅ 1/20th.

Added 1µl 10×PBS, heated to 85° for 3 min., iced
& to 80µl bacteria.

Also 80µl (≅5%) of crude B'152 M heated to 85° for 3 min, iced
& to 80µl bacteria

	Conc.d	Crude
DH5α B'133	80µl	80µl
DNA	11µl "10% M" B'157 heated	80µl B'152 M (crude) heated
Lce	6.22pm	to 6.53pm
42°	90secs	

Plated Rest, 20, 5, 20/16, 5/16
as 1X amp (Many) (11) (1)
as B'159 Crude
B'159 conc.d

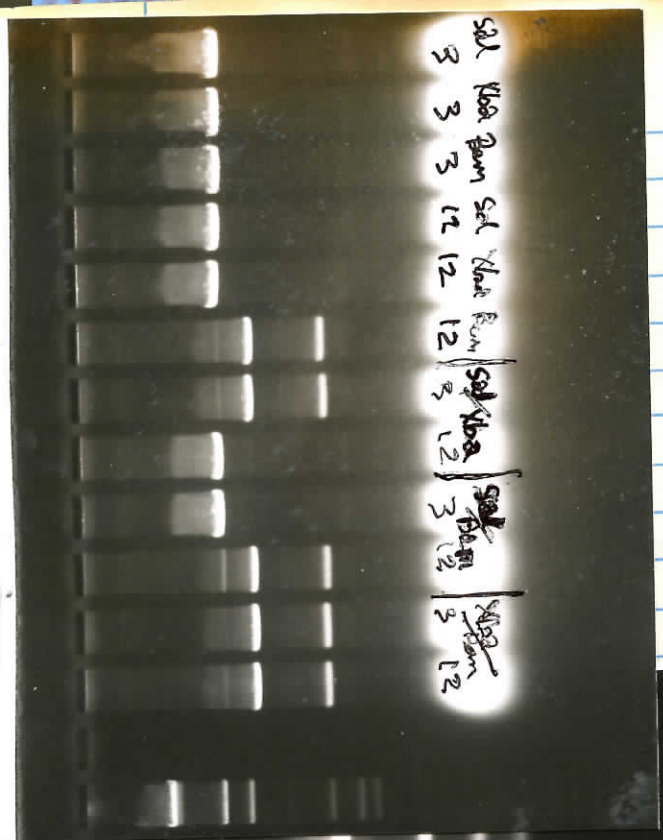
9:15am Mon. Zero on "Crude"
Excellent on "10% M"

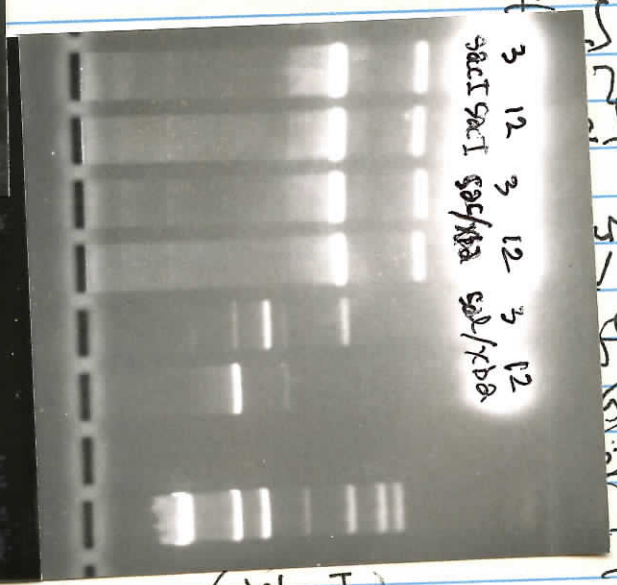
Mini B'159 #1—25 from ~10:30am to 7:30pm
with shaking. R.T. from then on.

Gel 2
0.8% HX4 MAX
6.45pm
~7.45pm

(2)
Dye
Water
1
2
3
4
5
6
7
12
W
M
W
Dye

B'159 XbaI Sal mini

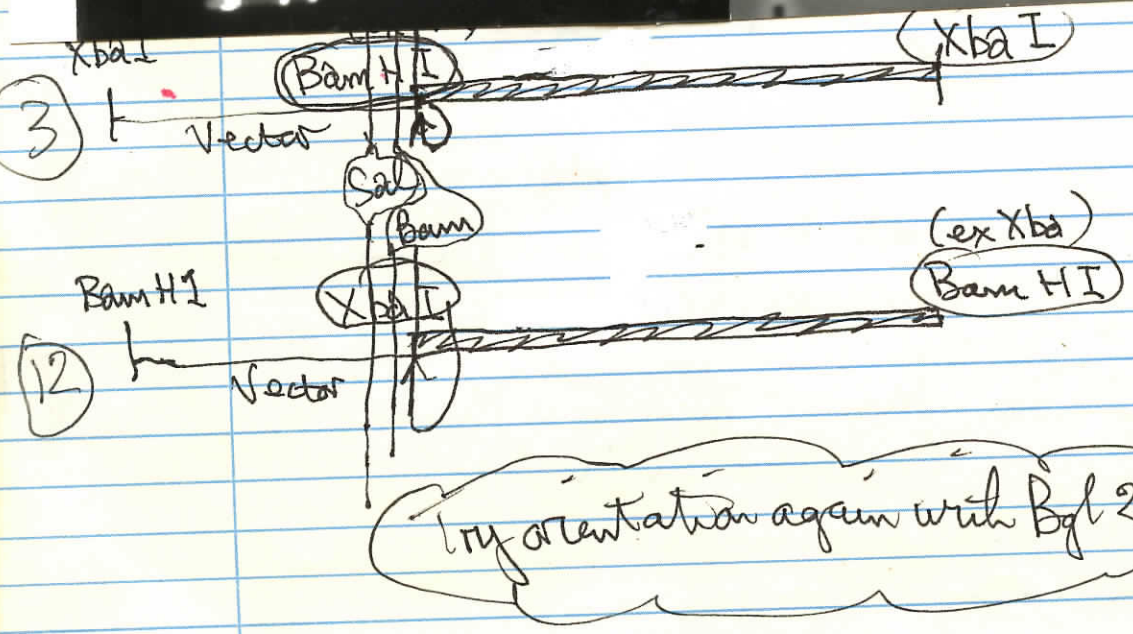




on 11.38am 12.54pm off 0.8% H14 max.

(1)

Dye
W
#3 Sal
#3 Xba
#3 Bam
#12 Sal
#12 Xba
#12 Bam
#3 } Sal/
#12 } Xba
#3 } Sal/
} Bam
} Xba/
} Bam
Dye
W
#3 } Sac I
#12 }
#3 } Sac I
#12 } Xba I
#3 Sal
#12 Xba
W
W
W
Dye



Try orientation again with Bgl 2

Wed. Nov 23rd

Checks on Xba ~~Xba~~ Bam

B'159 minis #3 & #12

M1 Take 30 µl 10x RS+1.5 MgCl2

✓ 270 µl ddw

S: 90 µl +1 µl Sal1 Lot 47 (1.5 µl) — 20, 20 → 1 µl #3 Sal, #12 Sal (no units)

X: 90 µl +1.5 µl Xba1 Lot 29 — 20, 20 → #3 Xba, #12 Xba

B: 90 µl +1.5 µl Bam H1 Lot 72 — 20, 20 → #3 Bam, #12 Bam

20, 20, 20, 20, 20, 20 → SX, SB, XB

SX: #3 SX, #12 SX (~3 units of each)

SB: #3 SB, #12 SB

XB: #3 XB, #12 XB

O.B. 1% max

37° 10:25am to 11:25am

Error - shit have used Sac I for orientation

B'159 #3

B'159 #12

streaked out 0 to 37° Noon Wed.

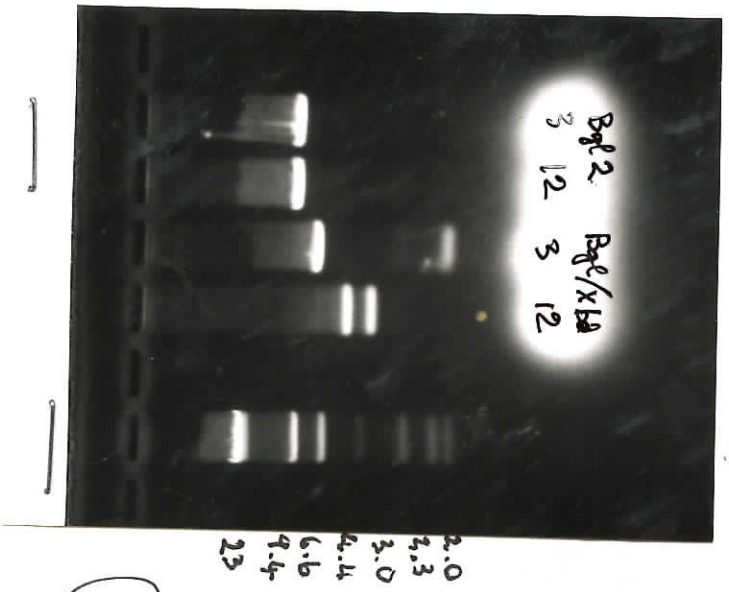
Need to recheck with Xba I and Sac I for orientation; Sal I was probably in the vector.

Take 10 µl R1 salts + 90 µl ddw + 1 µl Sac I Lot 45 — 20 µl → +1 µl #3 Sac, #12 Sac

50 µl +1 µl Xba I Lot 29 → #3 Xba Sac, #12 Xba Sac

37° 12:10 pm

1:30 pm



Thanksgiving Day, stained & photographed.

Conclusion: ← 2 → ← 3.5 →

#3 is Xba — Bgl — Bam ~~Xba~~

#12 is Bam ~~Xba~~ — Bgl — Xba

Final maps are :—

#3 B'159

XbaH1 — 2kb — Bgl2 — 3.5kb — BamH1 (ex. Xba) — 3kb — Xba1

This was the one planned. Can use for Xba — Bam; Xba — Sal

Bam — Sal1

#12 B'159

BamH1 (ex. Xba) — Bgl2 — Xba1 — BamH1

Can use for Bam — Xba; Bam — Sal

Bam — Sal1

on 7.05 pm
off ~6pm
0.8 g HX4 MOX

Wed - Nov. 23rd, 1994

Orientation of #3 & #12 B'159 (cont'd.)

Take ✓ 10 µl RO x R1 salts
✓ 90 µl ddw
✓ 1 µl Bgl II Lot 31

Rest +0.6 µl Xba I Lot 29

20 → #3 Bgl
20 → #12 Bgl
20 → #3 B/X
20 → #12 B/X

37° 6pm

Bye
W
#3 } Bgl2
#12 }
#3 } Bgl2 + Xba I
#12 }
W
Marker
W
Bye