

Chapel Hill

July 29th 2001

Dept. of Pathology

UNC-Chapel Hill

Chapel Hill

NC 27599-7525

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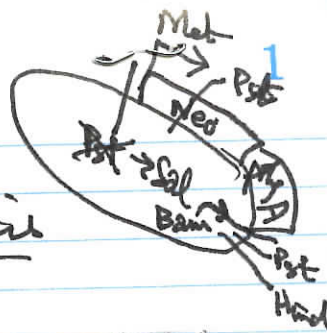
Contents

Page

[illegible]

Page # _____ Date _____
 Student Name: m2 Pst Gal Pst
 Signature: GAG ETC GAG ETC TGC
 Date: 29754
 Grade: A

Sunday July 29th
pMC1 Neo poly A modification
for NAACasept.



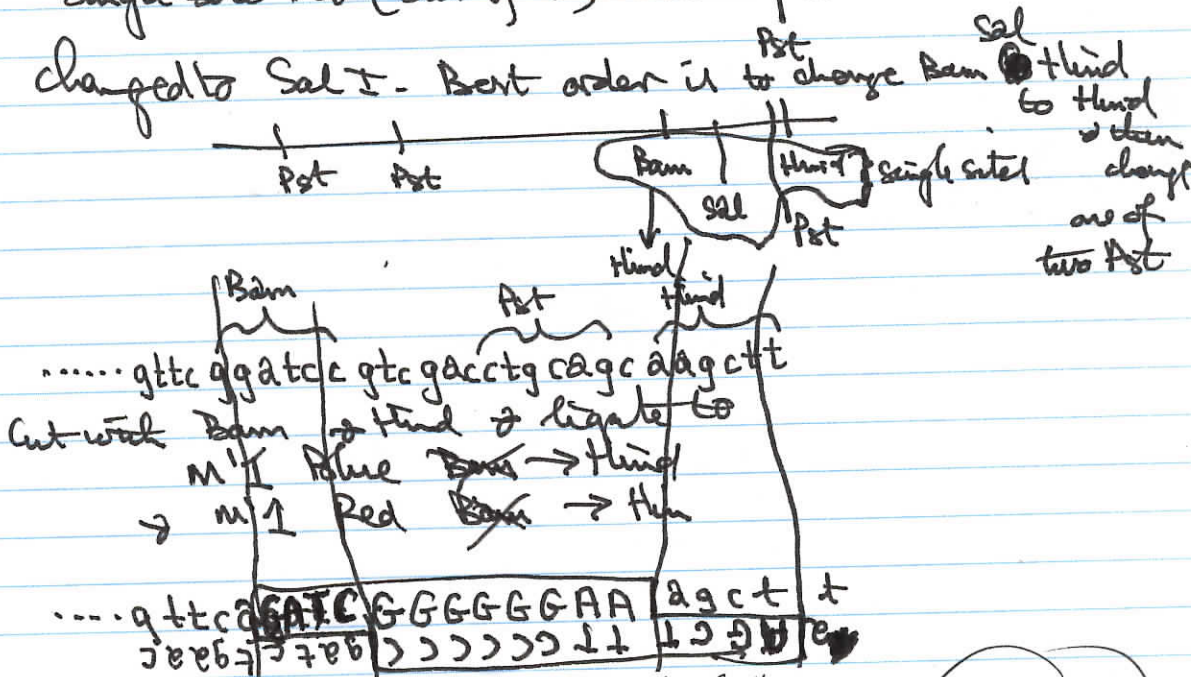
pMC1 poly A SHI 114 and KC AM1 pg. 158
 0.475 µg/µl

- now called M'1 pMC1 Neo poly A & kept with Dup Del
 Blue

Neo
 pMC1 good SHI 114 pMC1 neo good 0.505 µg/µl

- now called M'1 pMC1 Neo & kept with Dup Del
 Red

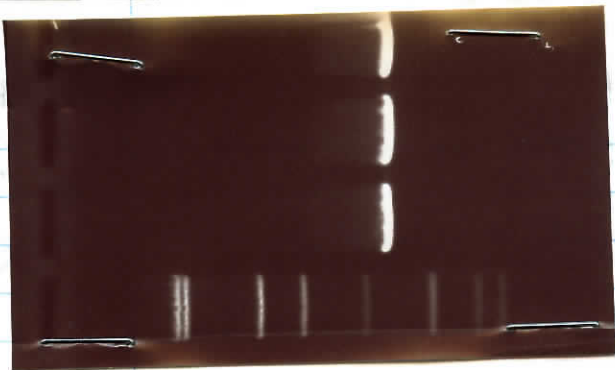
Single site Pst (out of 3) next to Pst met need to be
 changed to Sal I. Best order is to change Bam & Hind



Clon	Clon	Clon	Clon	Clon	Clon
29752	GAT	CGG	GGG	GAA	
LENGTH	12				
29753	AGC	TTT	CCC	CCC	

Continued
 M'3

2



① All enzymes worked & have single adjacent sites.



Cont. in M⁹

9:45 am
12 noon
0.61031
Sun day July 29th

Age (1) 204 (M'1 contol.)

Bam Bam/Hind Take 19 pe (200) pMC1 Neo poly A M'1

Hind 24 pe 10x RT salts

120 120 20 pe + 1 pe (200) Bam HI hot 80 (200/pe) Hind III hot 57 (200/pe)

M'3 Bam

M'3 pMC1 Neo Bam/Hind

M'3 Hind

37° 6:10 pm to 7:20

Frozen pending heat inactivation 80-85° 20 min

ligation 6 pe ddw Take 3 pe M'3 pMC1 Neo Bam/Hind

M'3 Neo Hind hlg

4 pe M'1 Bam → Hind
4 pe M'1 Red Bam → Hind
2 pe 10x p95 hlg⁺ buffer
1 pe K'99 T4 ligase

Tue 15 10:46 am to 3:10 pm, RT 2:10 pm to 3:40 pm, 37° 3:45 pm to 5:40 pm

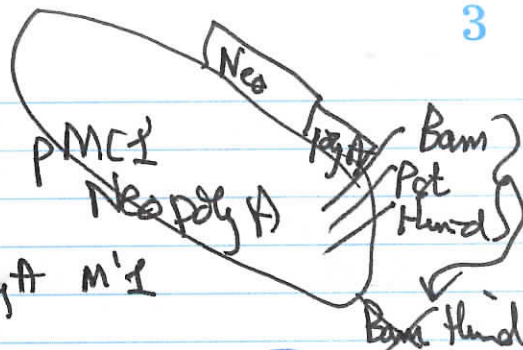
T.H. will continue.

Transformation

DH5α 80 pe
M'3 hlg⁺ 3 pe R, 20, 5, 20/16, 5/16.
0° to 90 sec 42°

24 M'3 grown & tested 23/24 appear correct *

* → (14) (17) (20) were tested by T.H. - have correct pattern after Pst, i.e. 200bp + 3.7 kb vs 200, 700, & 3 kb. Still intact Hind



See M'5
M'3 BGBP

Product check with Hind ✓ Yes
Bam X No

OK for BMHC 190.8g 7/27/#1 Blue (3) Blue M/V K17 L12

OK for 191.8g 7/27/#2 Red (1) Blue M/V

OK for BMHC 192.8g 7/27/#3 Blue (3) Blue M/V

193.8g 7/27/#4 Red (24) Blue M/V

OK for PURO Poly A 194.8g 7/27/#5 Blue (3) Red A/V

OK for PURO Poly A 195.8g 7/27/#6 Red (4) Red A/V

Tues. July 31st

See M'43

Blue 3 & 13, Red 4 & 24

Blue
K17 M10 }

L121 Red
AOT }

Blue 3
BMHC hrGN.BMHC PURO

Conclusion:

N154 HWB BMHC PURO

L155
Blue (3)

Red (4)
L155

Red 4
Renamed

M'3
BG BP

Both

Correct
Renamed

M'3
G BBP

Blue (13) is also OK

In computer

Faded
probably
too little DNA

→

B G

→ { Added
Later

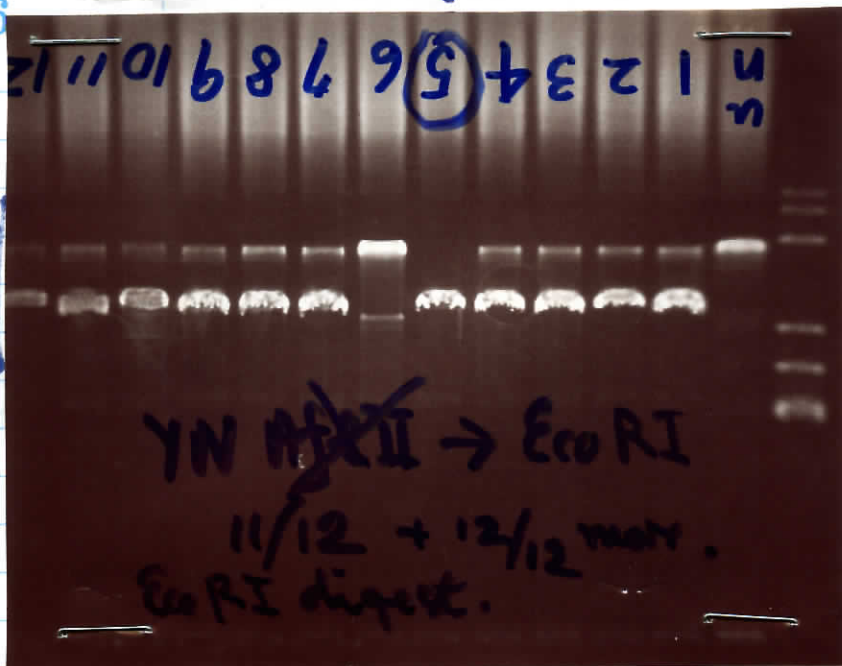
B P

BMHC hrGN BMHC PURO

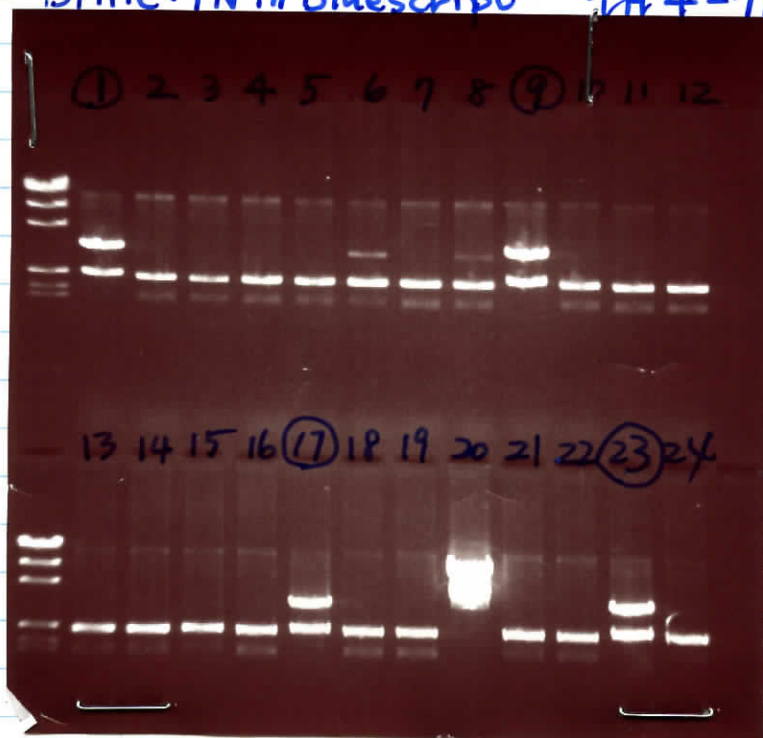
into HPRT → HAT

Seig has done this - see M'43
it is making more ES cells.

6



BPHC-YN in Bluescript TH 4-71



Tues. July 31st

New strategy, son

Use a linker that destroys the
Afl II site. ligate, then redigest with
Afl II $\rightarrow$ CIAAP prior to transformation.

T. H. suggested removing the phosphate in
CIAAP the Afl II digested piece & make the
linker. She did this with spectacular
success: 23/24! Pick #5 as

M'x
YN
Eco 5

Fragment made by TH/O.S.
now called (XVI)

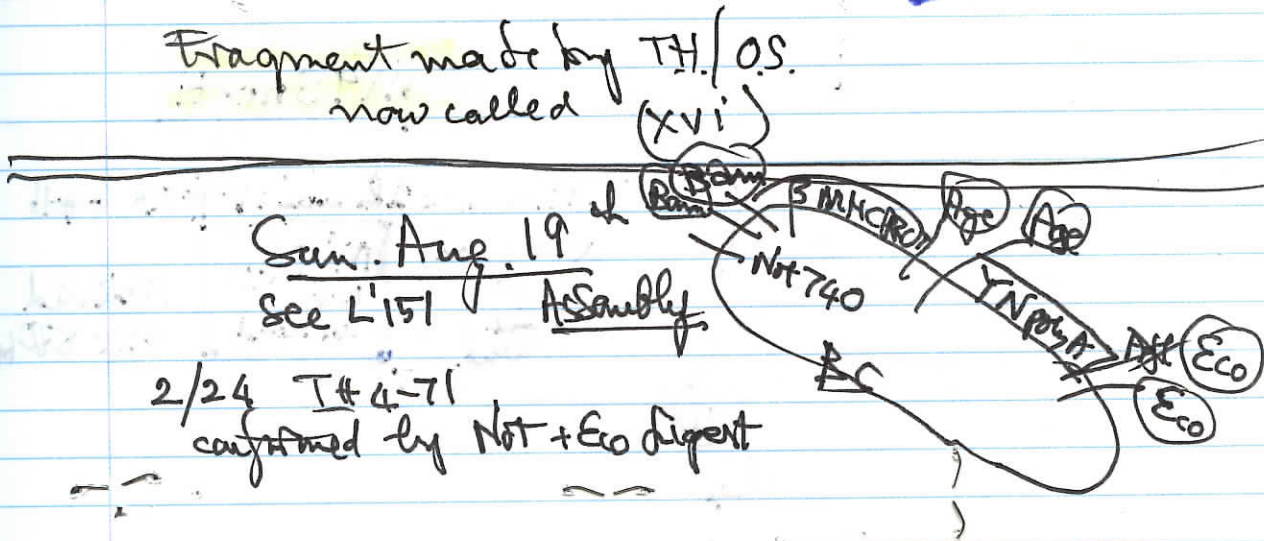
Sun. Aug. 19

See L'151

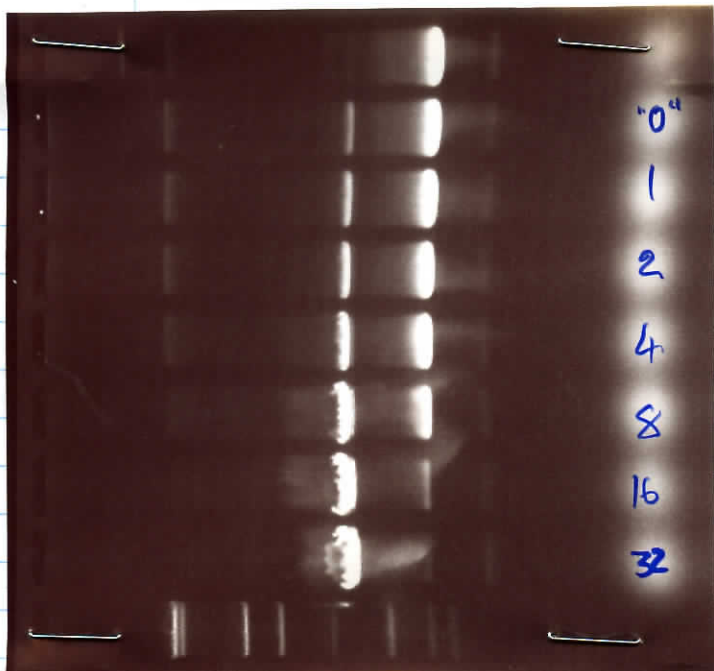
Assembly

2/24 T#4-71

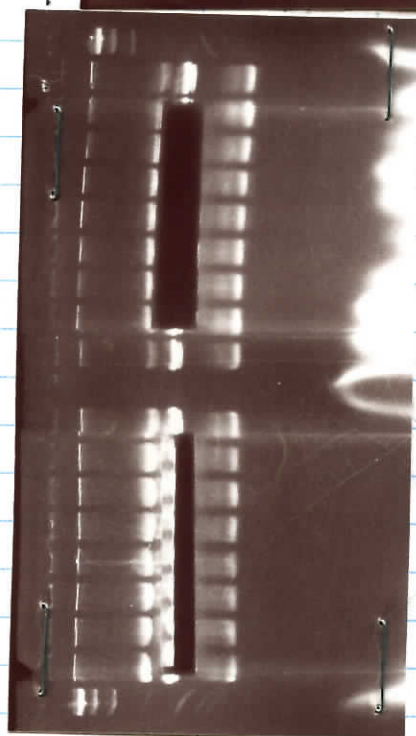
confirmed by Not + Eco digest



8



① Pool 4, 8 & 16 for prep. gel as M'9 Pool ①
 Digestion was rather short.
 Pool "0", 1 & 2, added
 returned to 37°, added
 1/2 µl Pst & stopped after
 3 min ✓. Use as
 second pool for prep
 gel M'9 Pool ②



② The two pools are ~ equivalent. Pooled
 & recovered into 100 µl at M'9 PMcINeo frag
 Pst 5 (single) (at)

M'9 SneoH

T.H. made 1/2 minis ✓ #23 off 16
 are good.

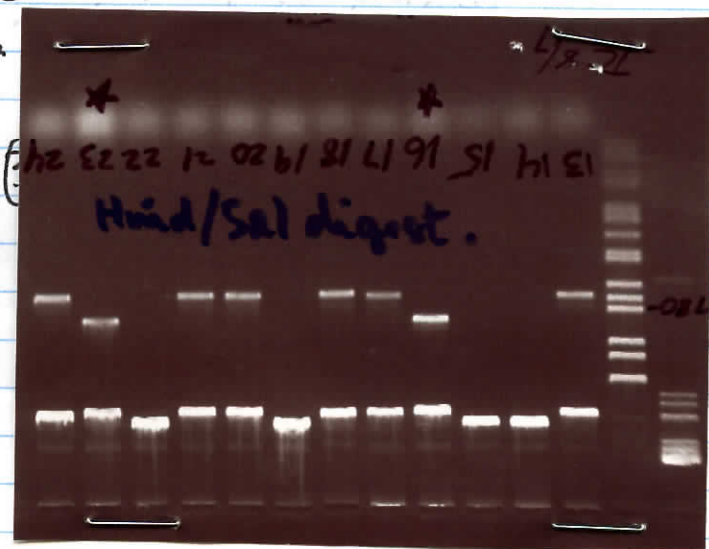
Continue with

M'9 (16)
 Neo
 Sal Hind

Practiced
 size 880 bp



③ Good product. Proceed.



oe (i)
heart.

ed
ter

ing
(at)

-16

ed
880 bp

ed
880 bp

ed

11:30am
0.86 Hx4
3/10 RNAse

① Dye
W
Control
"0"
1 Sample cut Pst
2
4
8
16
32
AGMM
W
Dye

Fr. Aug 3rd
Single site cut for
Pst $\rightarrow$ Sal and Pst $\rightarrow$ Sal Pst

Take 120 μ l of T.H. pooled minis 11, 12, 13, 14, 17
✓ 40 μ l of 10x R1 salts
✓ 240 μ l of ddw
Warm to 32°
Add 1 μ l (200) Pst I lot 39 (200/ μ l)
M³
Proven OK.

Control 20 μ l 60 μ l samples
"0", 1, 2, 4, 8, 16, 32, Pst

Inactivate $\geq 80^\circ \geq 20$ min

② 11:15 pm
11:45 am
0.86 Hx4
1/10 RNAse

Dye
AGMM
9 slots of M⁹ Pool ①
Dye
8 slots of M⁹ Pool ②
AGMM
Dye

③ 0.86 Hx4
Max

Dye
W
AGMM
W
M⁹ pMCINeo Pst sc. frag
W
Dye

Take
10 μ l ddw
✓ 3 μ l M⁹ pMCINeo Pst sc. frag
✓ 4 μ l M⁹ Pst Sal Pst oligo
✓ 2 μ l 10x p95 mt. buffer
✓ 1 μ l R⁹⁹ T4 ligase
M⁹
Neo
Sal
ligase
15:20 pm
15:50 pm
Sat.

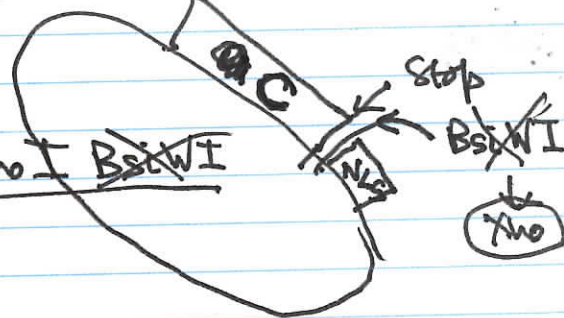
Colt room 8 pm to 4 pm
Sat. Sun

Transformation
3/13 PH54 TH6/8 80 μ l
M⁹ ligase 3 μ l
0° 4 pm to 4:40 pm
90 sec 42°
Plated 1x AMP NZT 3 to 5 pm
R. 20, 5, 20/16, 5/16
Many

Doris Sykes says!

Thurs. Aug 9th

C BsiWI → ~~BsiWI~~ XhoI ~~BsiWI~~



Start with L'27 (C) 23

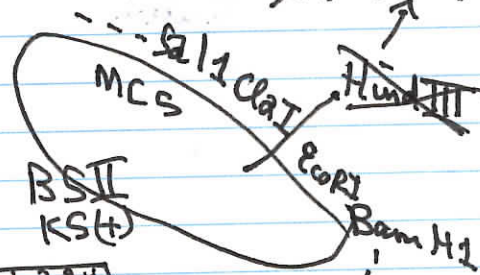
Digest with BsiWI. Heat inactivate at 80° for 20 min.
Then use oligo TH4-61 ~~Bsi~~ Xho ~~Bsi~~
TH will do this. TH4-62 #9 was correct &

is now M'11 (C) ~~Bsi~~ Xho ← qian
XhoI #9
TH4-62

Error This has no poly A.

Tues. Aug 7th

TH4-59 17/24 with oligo
Not T → X
a ATGC TGG ACT TAA GTC C
53 138 111 54 33 1338

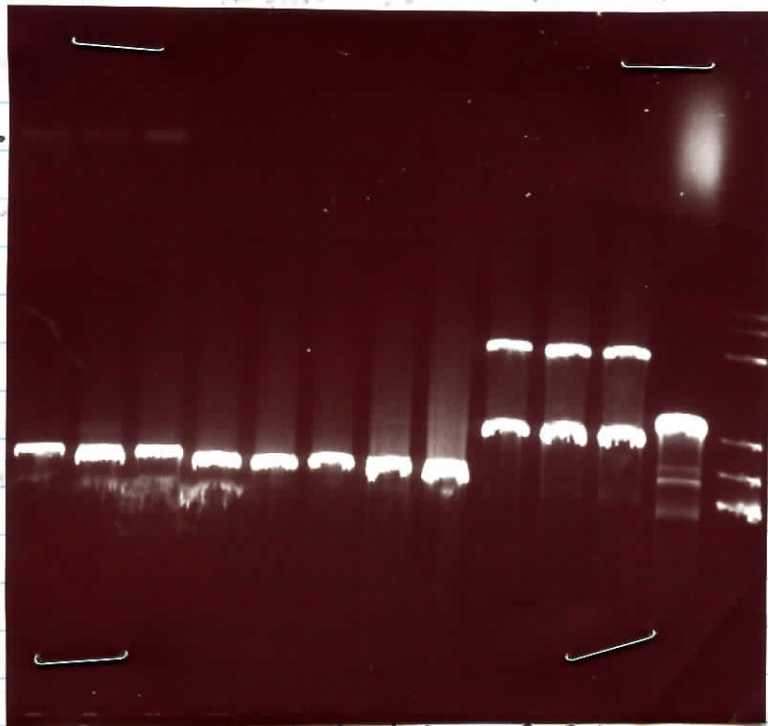


M'11
BSAseII ← see TH4-59

12

~300bp
→

→
Note
diff



3 2 1 | 3 2 1 | 3 2 1 |
AatII Age I Start AatII Barn Vncut m'13 x MHC Age
from Start
cloned clone
cut with Aat II

TH4-90
later comment.

Found 2 Age sites
in modified MHC
clone - close together.
Sh'd not affect final
constructs, but
need to see that
the extra 300 bp
are not in the
construct
(see m'29)



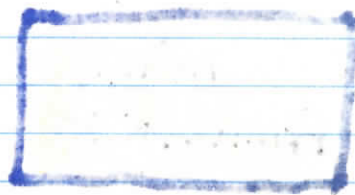
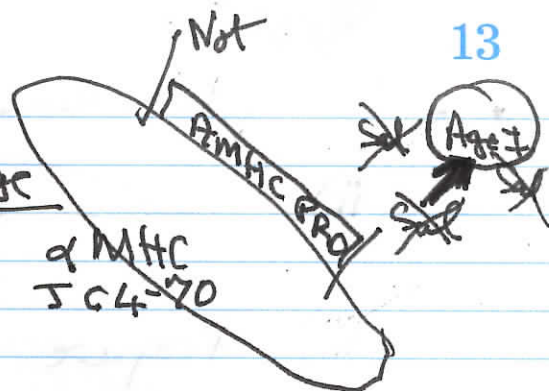
Thurs. Aug 9th
AMHC PRO Sel → Age

αMHC rec'd from Jim Guelick
 @Diagen prep 7/23/01 (L'144). Now
 called **M'13 αMHC**.

αMHC
 J C 4-70

J.C. will try this with T.H.

Dorely J.C. **M'13 αMHC Age**
 J C 4-75



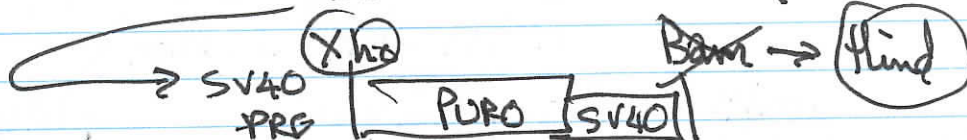
14

Steps:-

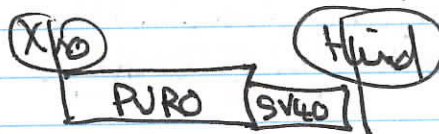
i) pPUR Clontech



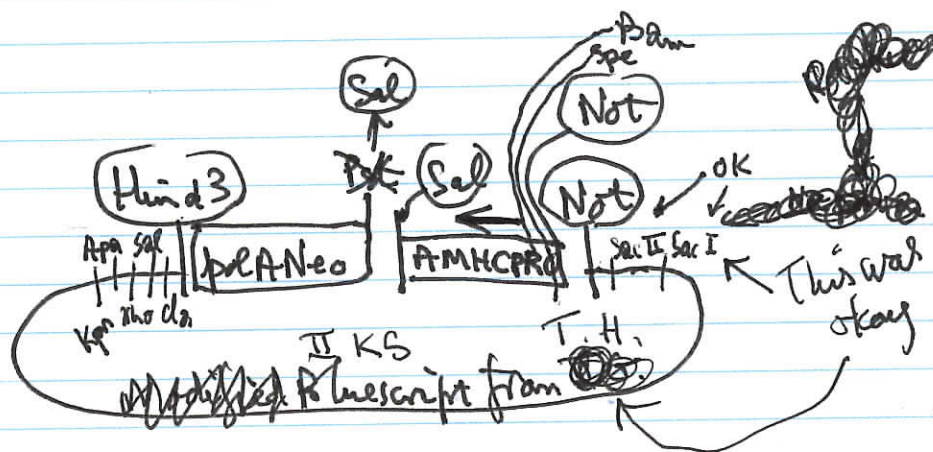
ii)



iii) Prepare



M'14.
AMHC.NEO



Made
Aug 31st, 2001

TH4-83

See M'94 for

M94 AMHC.NEO

Sat. Aug 11th

New thought

The BMHC PRO driving hTGN and PURO allows selection for fetal type cells.

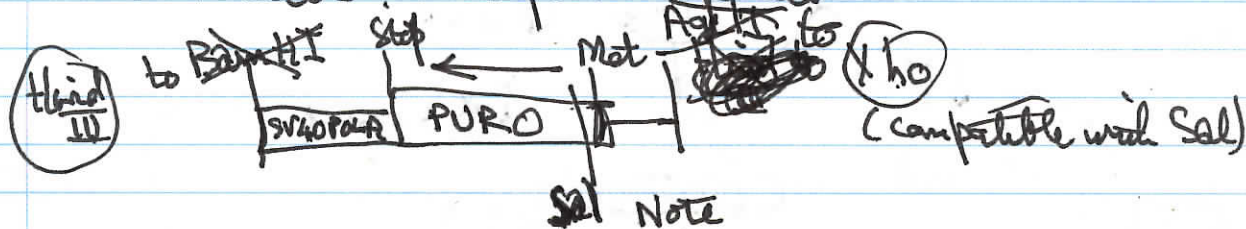
The 2MHC PRO driving C and Neo allows selection for adult type cells.

I need a means of selecting for fetal OR adult and/or fetal AND adult.

A simple solution is to replace Neo in the 2 group by PURO.

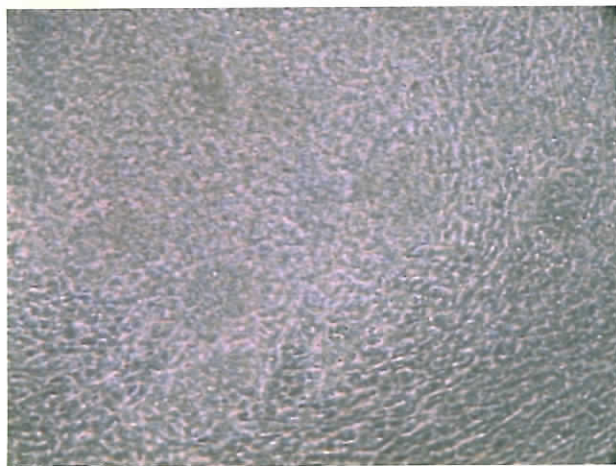
Referring back to L'147 NAAC omega plan, the

(Hind) pA Neo (Sal) piece needs to be replaced by (Hind) pA PURO (Sal), if this can be made. Simplest approach to be

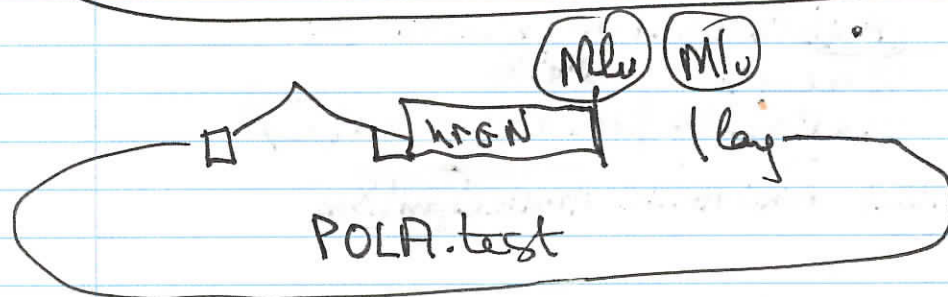
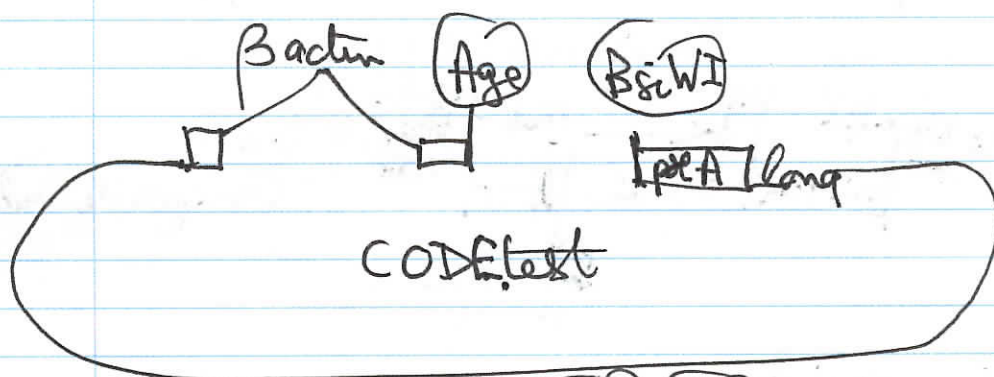
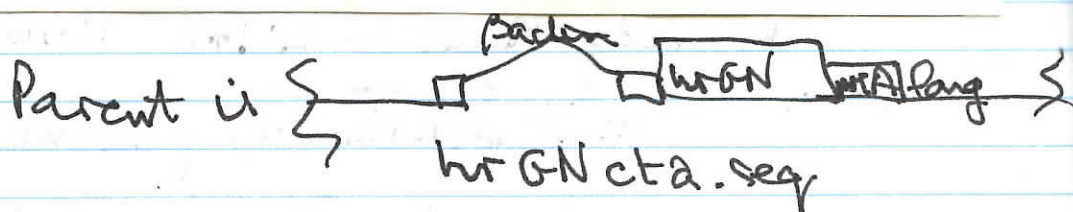
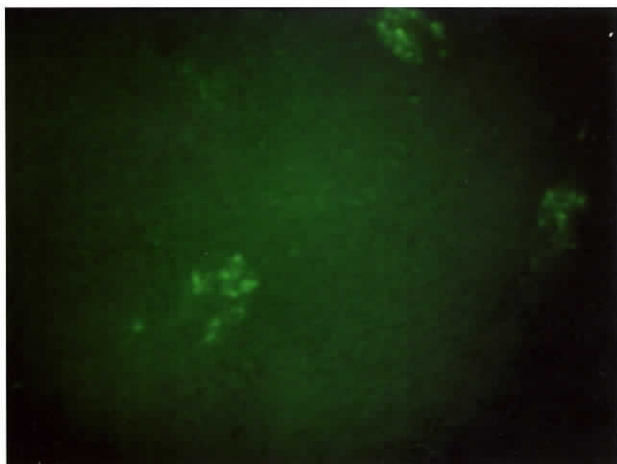


Note (Hind) SV40 PTA PURO / AMHC PRO (Not) will need to be assembled first before making (Hind) PURO AMHC AMHC Cyan (Xho)

Bright Field



Fluorescence



Wed. Aug 29th

BMHC PRO. PURO

Joe reports & showed me BMHC. PURO cells still
beating after 2nd days in puromycin & general cell death.
Ditto Sat. Sep 1st Tues. Sep 4th after trypan

later comment
Not as bright as Bactin hr GN
by a big factor

Sun. Aug 19th

L119 construct

17

BMHC PRO. hr GN

in HPRT locus

Joe / Seigo report ES cells gave beating
cells that are nicely green! ~~many more~~ that are
beating & not green.
It is working!!

The other cells in culture do not fluoresce.

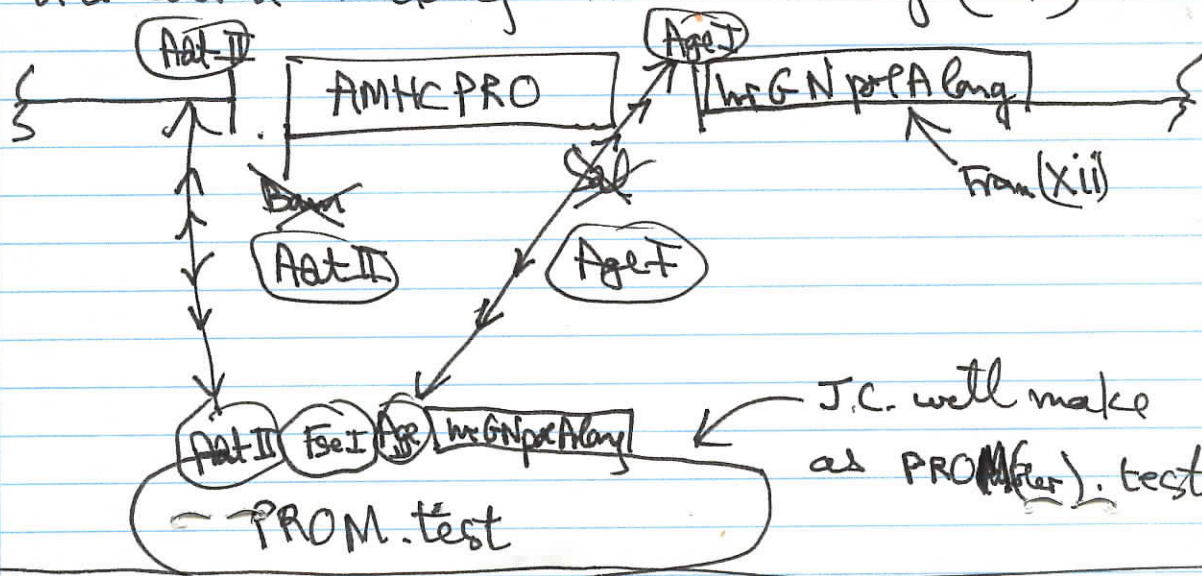
β

Seigo will inject to
derive animals

Mon. Aug 20th

5' homolog. AMHC PRO. hr GN cta 3' homolog

In view of low frequency of BMHC myocytes,
it is worth making AMHC. hr GN long (XIX)

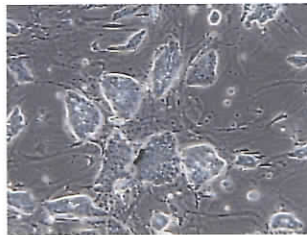
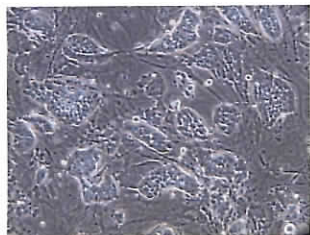


death.
try pin

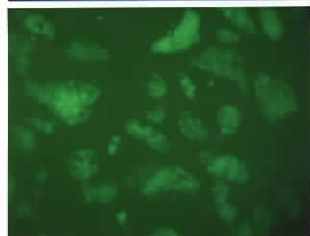
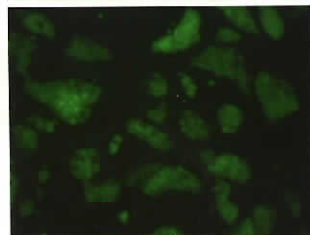
Some small colonies (?) are still alive & beating. Not much else is alive. Single cell beating ~ only 1 nucleus!

hrGNCTA

CCTA



Bright field

Cyan filter
XF114-2Green filter
XF100Yellow filter
XF104-2Red filter
XF102

Exposing time: 0.5 sec.

XF104-2

XF114-2

Wed. Aug 22nd

BMHC hr G-N / AMH C.C

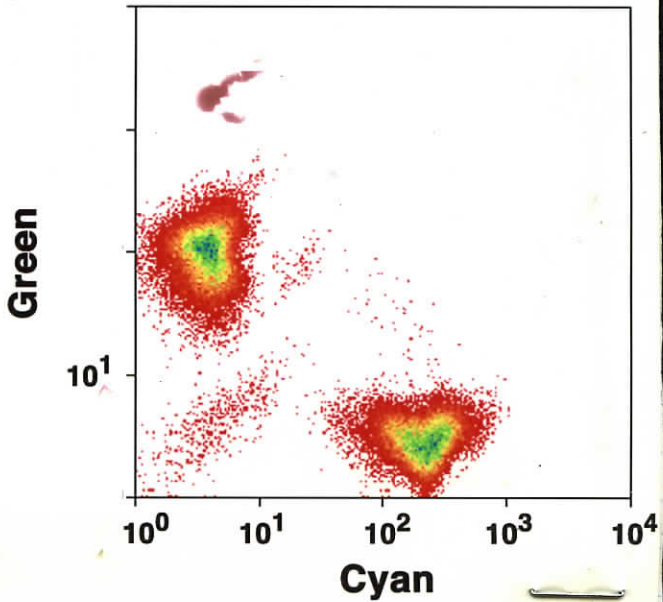
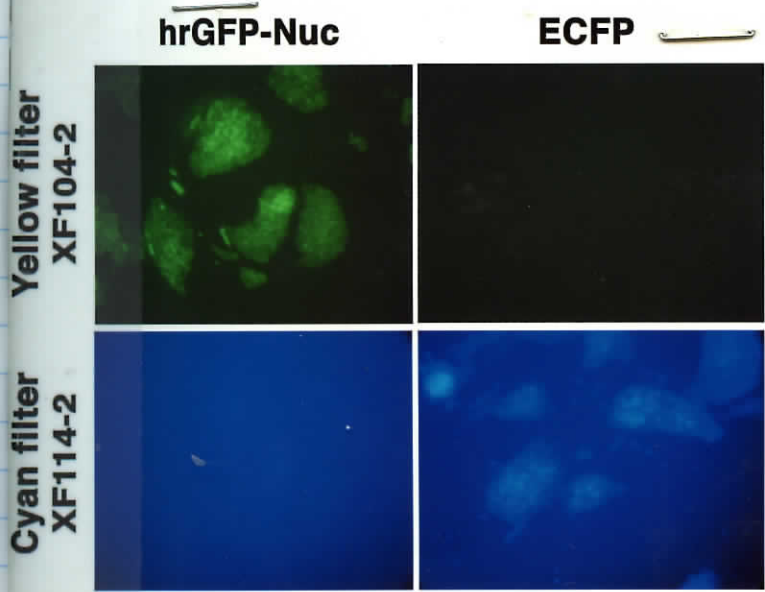
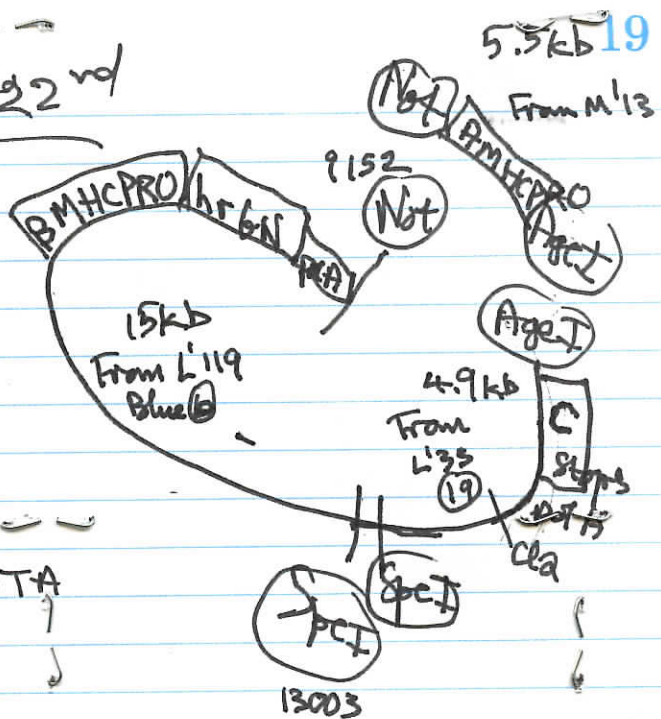
M'16 The result with BMHC hr G-N prompts me to make the simplest double:

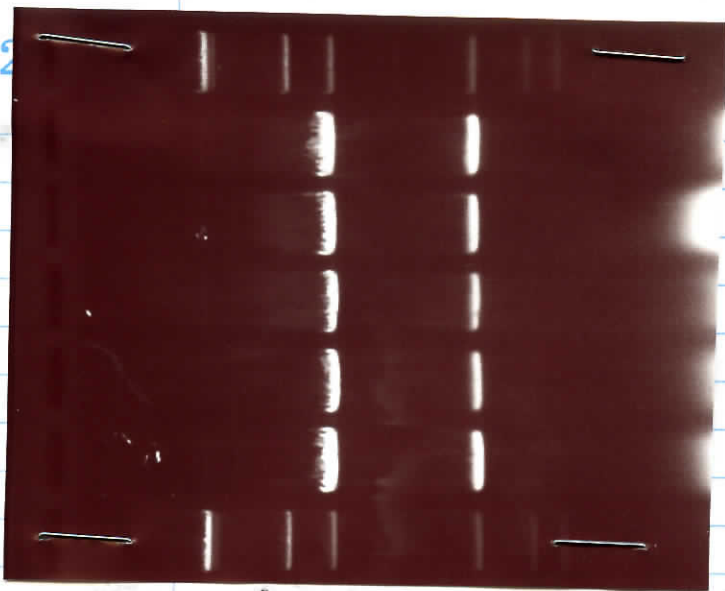
M'18 The results with hr G-N CTA and C CTA

show that three filter sets will allow either, or, and discrimination.

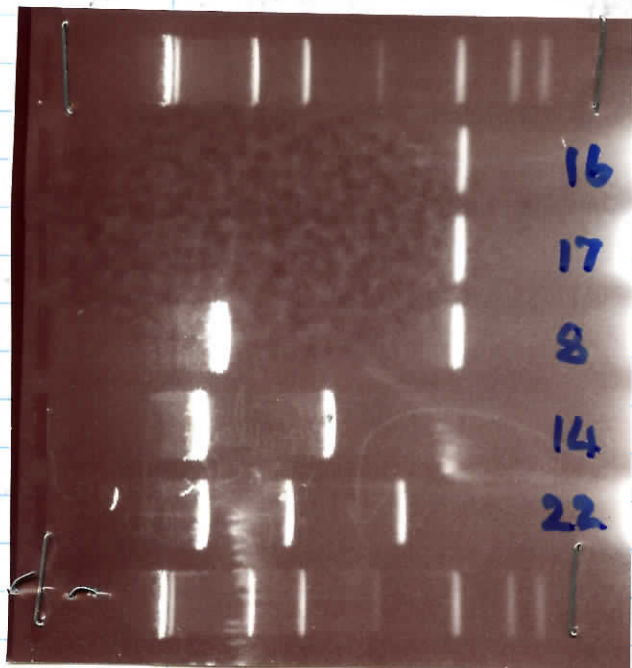
late November

Excellent FACS cooperation of ES cells





① All correct.



② #22 looks very good.

First sequence assembly says
Spe⁺ | Not Not Not Not Spe⁺

(4.6 kb), (6.5 kb), (3.8 kb)

↑ ↑ ↑
Obs. ~7 kb ~4 kb

Since linkers are missing, this
looks fine. Check against
input fragments with

Not+Spe; Spe+Age; Age+Not or I perhaps
all 3 sh'd work except for extra Not.

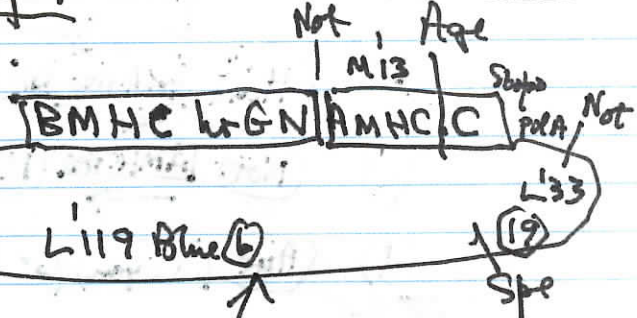
Sat. Sep. 1st

Tue. Oct. 2nd Using m'33 virus
Blue beating can find
myocytes observed!!!
2 areas

Relief from grant writing!

0.8% H4 max
0.2% H4 max
0.1% H4 max
0.1% H4 max

TH got 3 candidates for



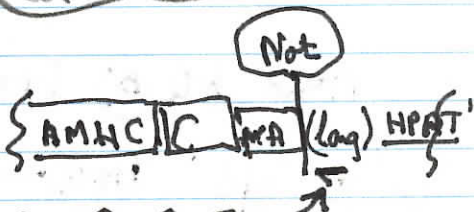
2 digested with Not & Spe.

Agarose	(2)
W	
AGMM	4-84
16	TH Not Spe
17	digests
8	for L'119
14	
22	
AGMM	
W	
Agarose	

Not Hind
for M'14
AMHC.NEO

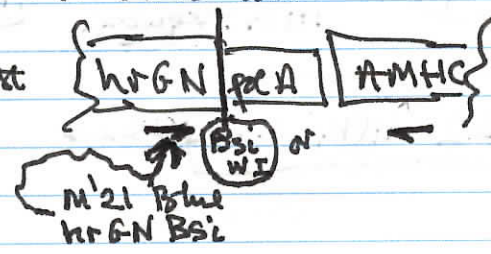
M21.BhrGN.AC

This has an error
corrected M'33



Sequence check K'17 M'10 will check 3' end of cyan & pA

Make a probe to test



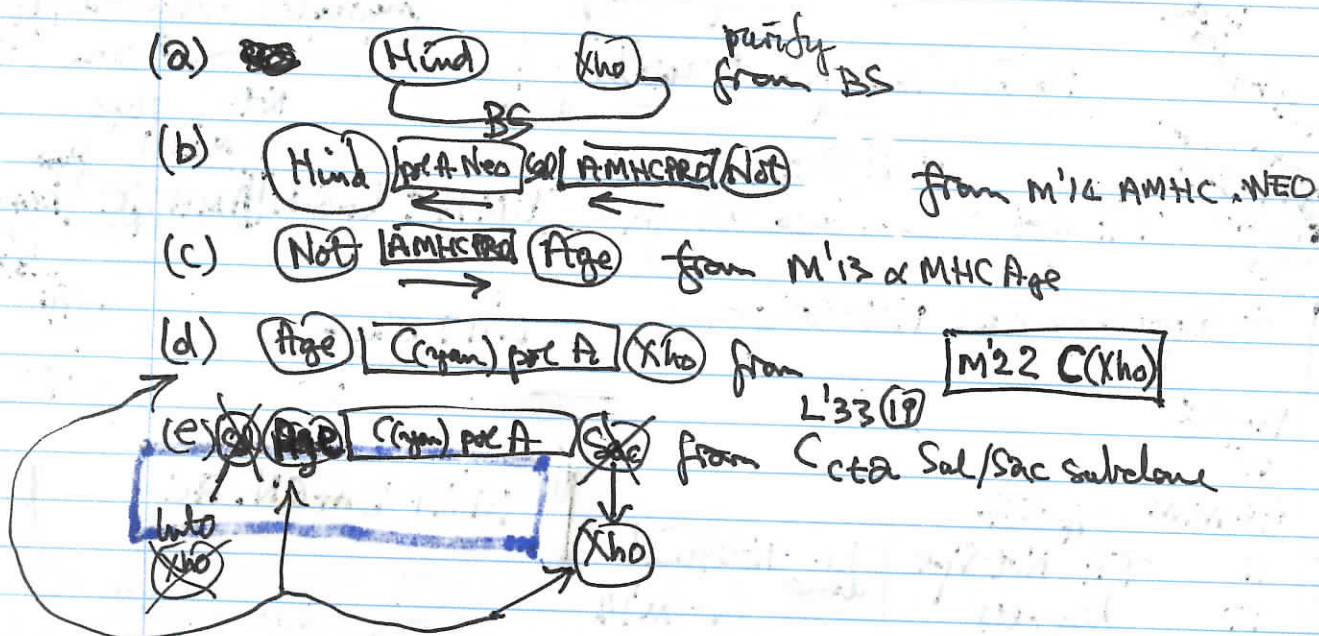
Assume this is correct.
Go for targeting.
Go for animal.
Cancel BHRG.BhrGN animal

Continued on M'27

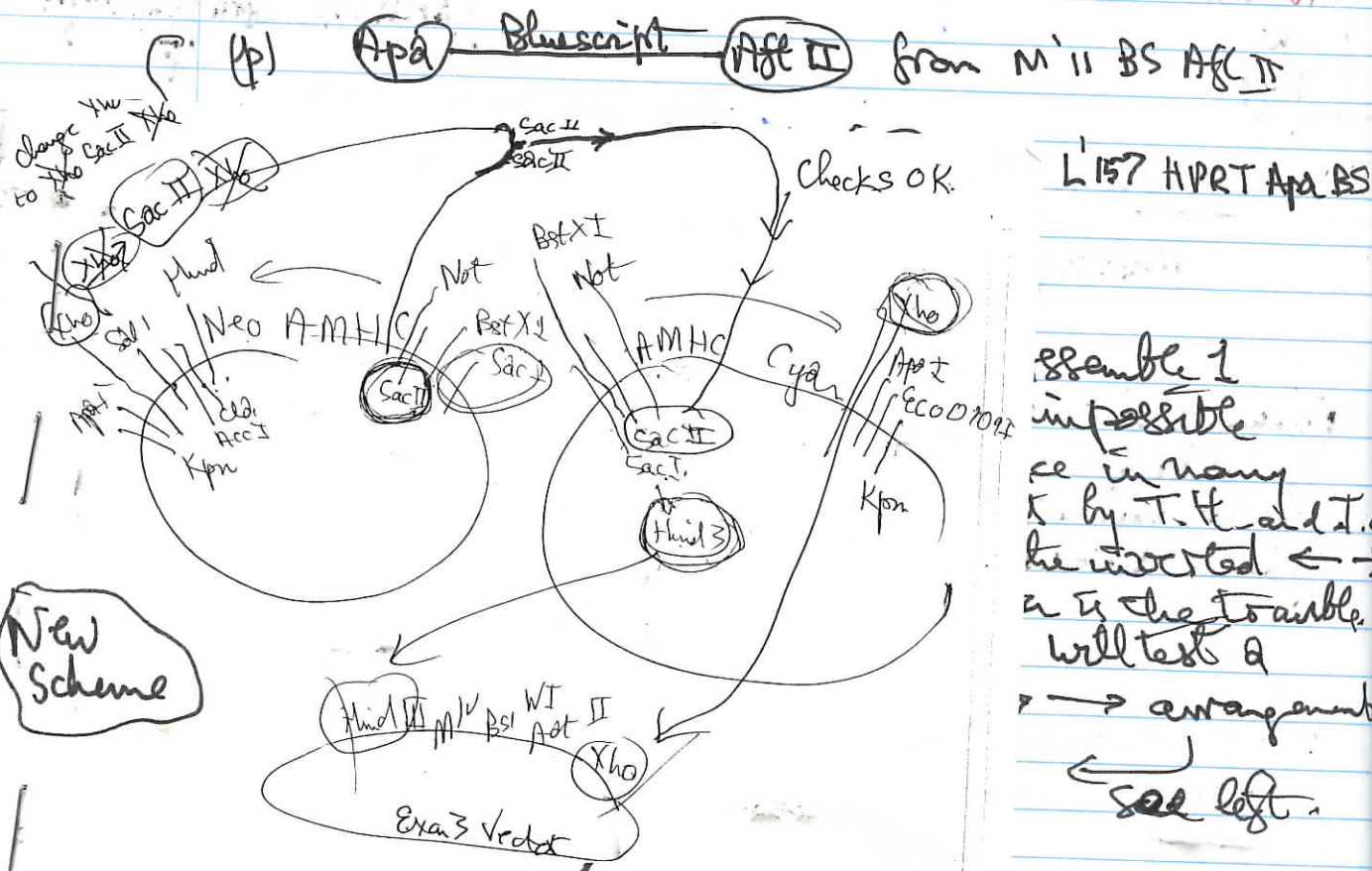
30213	M'21 Blue hrGN Bsi
GTG	TGA
TAA	GGA
TAC	GTA
C	
870	8760
8761	8820
8821	8880
8881	8940
8941	9000

40

Assemble (1) from :-



Assemble (2) from :-

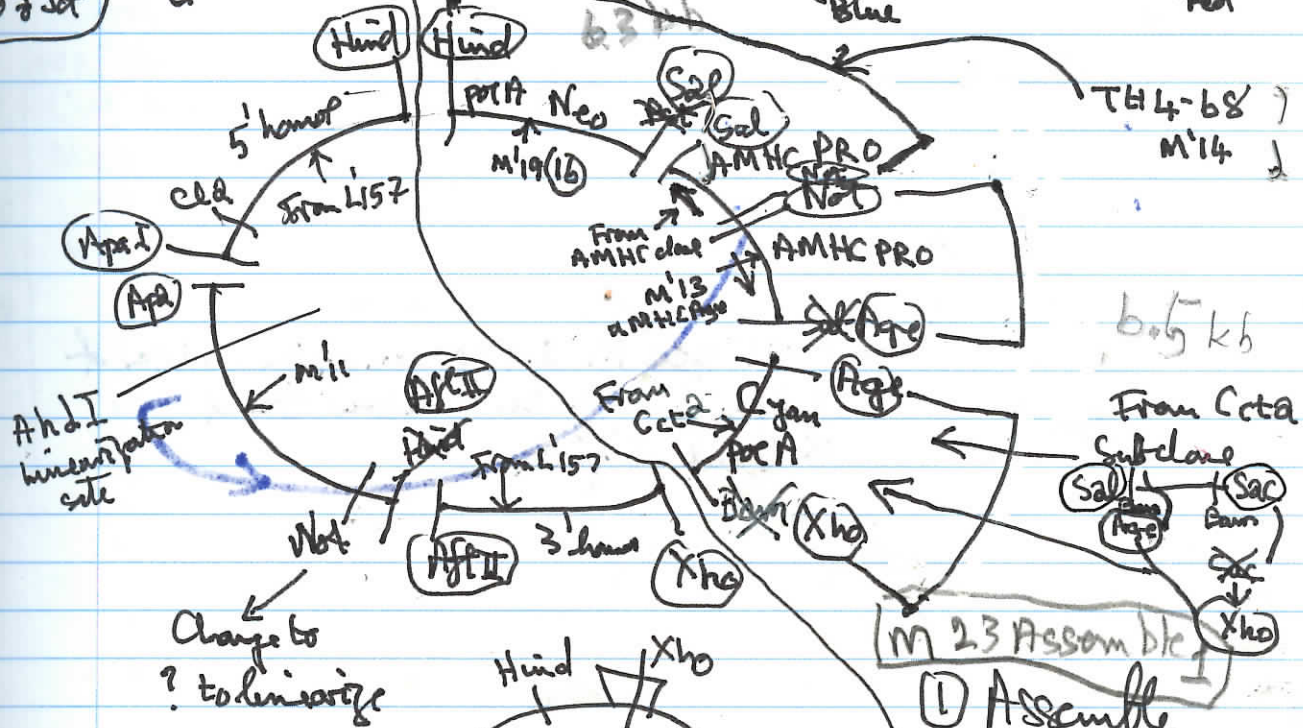


Sunday Sep 2nd

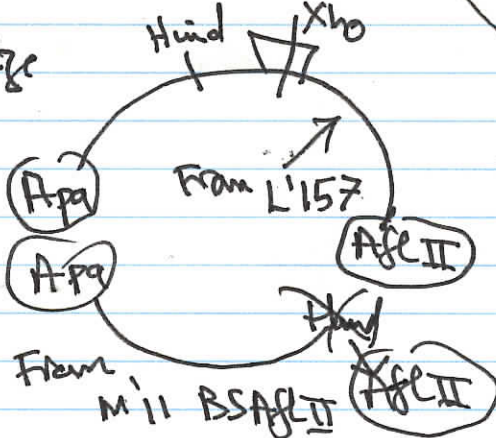
Review of status of assemblies

The AMHC Neo AMHC Gen omega is highest priority for testing Neo selection of α and double with β puro selection. AMHC Puro AMHC Gen is a close second. BMHC Puro BMHC h6N ~~is~~ worked & is ~~not~~ under test * as M'3 BGBP or M'3 GBBP - Red

* Check with Sings & Jot



(2) Assemble from

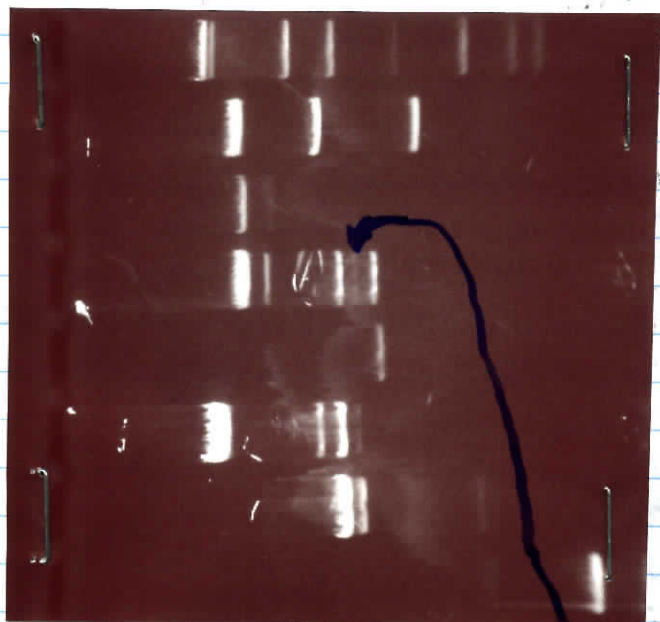


(3) Excise Hind-Xho from (1) & put into Hind-Xho from (2).

(1) Assemble

as one fragt.
with 2 + piece
ligation using
(Hind) (Xho)
BS

as vector (2)



(1) $\frac{Age-Spr}{2}$ be 4.9 $\frac{Not-Age}{2}$ 5.5 $\frac{Spr-Not}{2}$ 14.6

These are very close. Proceed.
There will be a ~~check~~ check
when sequencing data are seen.

Point see M' 29 for an explanation of
this band & of the shadow band
in total digest.

Memorial Day ~~WW II~~ began in U.K. 25

Thurs. Sep. 30

Checks (M²¹) on B h G. AC (yan) (#22)

2 hrs. 37⁰

① 6:12 AM
6:20 AM
H x 4 W x 3/10
H x 4 W x 3/10
H x 4 W x 3/10

T.H. digested/wired the pairs & provided comparative frag.^{ts}

Age

W

ACMM

M²¹ Not Spe digest (L¹¹⁹ AMHC)

L¹¹⁹ Not Spe frag^t

M²¹ Age spe digest (L¹¹⁹ AMHC)

L³³ Cyan Age spe frag^t

M²¹ Not Age digest (L¹¹⁹ AMHC)

Not Age frag^t | AMHC Not Spe

ACMM

W

byr

T.H. Label

6+10

6+10

6+10

Begins in hrGN, continued to Not I, then picks up AMHC promoter as designed.

5' end of C(yan) is fine, including stop & pA for hrGN

196

196.seq check: 2834 from: 1 to: 832
/afs/isis.unc.edu/home/n/o/nobuyo/oliver/hrGNuc.seq check: 6478 from: 1628
to: 4271
aps: 0 Quality: 2258 Ratio: 9.408 Score: 234 Width: 24 Limits: +/-25

1 GTNCCGGNCNTAATCAGCCATACCACATTTGTAGAGGTTTACTTGCTT 50
1629 GTTCATGATCAATCAGCCATACCACATTTGTAGAGGTTTACTTGCTT 1678

51 TAAAAAACCCTCCACACCTCCCCCTGAACCTGAAACATATAATGAATGCA 100
1679 TAAAAAACCCTCCACACCTCCCCCTGAACCTGAAACATATAATGAATGCA 1728

101 ATTGTTGTTGTTAACTGTTTATTGCGGCTTATAATGTTTCAATAAAG 150
1729 ATTGTTGTTGTTAACTGTTTATTGCGGCTTATAATGTTTCAATAAAG 1778

151 CAATAGCATCACAAATTTACAAATAAAGCATTTTTTCACTGCATTCTA 200
171 9827 AGCATCACAAATTTACAAATAAAGCATTTTTTCACTGCATTCTA 9876

205 TGGTTTGTCCAAATGATGATCTTATCATGCTCGATCCCTCGAA 254
18: 9877 TGGTTTGTCCAAACTCATCAATGTATCTTATCATGCTCGATCCCTCGAA 9926

255 GCTAATTCGAGCTCGGTACGACGCGTCTATGCGGCCGCTCTAGAACTAGGATCCT 312
9927 GCTAATTCGAGCTCGGTACGACGCGTCTATGCGGCCGCGGAGAAATTCT ggatcct 11110

313 GCAAGGTCACACAAGGGTCTCCACCCACAGGTGCCCTAGTCTCAATTC 362
11111 gcaaggtcacacaaggggtctccaccaccaggtgccctagtctcaatttc 11160

363 AGTTTCCATGCCTTGTCTCACAATGCTGGCCTCCCGAGCTAATTTGG 412
11161 agtttccatgccttgttctcacaatgctggcctcccgagctaatTTgg 11210

413 ACTTTGTTTATTCAAAAGGCGCTGATGCGAGTAGATCTTGCTA 462
11211 actttgtttttatttcaaaagggcctgaatgaggagtagatcttTgcta 11260

463 CCCAGCTCTAAGGGTGCCCGTGAAGCCCTCAGACCTGGAGCCTTTGCAAC 512
11261 cccagctctaagggtgcccgtaagccctcagacctggagcctttTgaac 11310

513 AGCCCTTTAGGTGGAAGCAGAATAAAGCAATTTTCTTAAAGCCAAATC 562

AMHC PRO

water

Thurs. Sep. 6th

27

M'21 BMHC h₆N AMHCC (cyan)

See M'29
M'33
Corrected

196.seq
09/03#2

Blue h₆N Bsi M'21 primer

M'21 BMHC h₆N AMHCC (cyan)

Inverted

197.seq
09/03#1

K'17 Red Mlu primer

M'21 BMHC h₆N AMHCC (cyan)

All essential parts are confirmed. Proceed with Zap & injections.

* Caution!! some indication of problem at 3' end of AMHCC. *

197.seq check: 9089 from: 1 to: 846
/afs/isis.unc.edu/home/n/o/nobuyo/oliver/Ccta.seq check: 3487 fr
19236
ASSEMBLE September 2, 2001 13:41
saps: 7 Quality: 6720 Ratio: 7.981 Score: 558 Width: 14 Lin

5 CCNTGCTNGGGGG...NNGGTTNANNCNTCCCGNCCNANANGAAA 50
9094 CCCTGACCTGGGCGTGCACTGCTTCAGCCGCTACCCCGACCACATGAAG 9143
51 CANGCNGA.TTTTNAAGGCCCGCATGGCCNAAGGGTNANGNCAAGAGGG 99
9144 CAGCACGACTTCTCAAGTCCGCCATGCCGAAGGCTACGTCCAGGAGCG 9193
100 GNCNTTTTTTCAAGGANGGCGNAANTTCAAAAANCCGCGCCNAAGTTA 149
9194 CACCATCTCTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGA 9243
150 AGTT.NAAGGNCACNCCCGGNSAACCCNATNAAATANGNGGGGGNTGAC 198
9244 AGTTCGAGGGCGACACCCTGGTGAACCGCATCAGCTGAAGGGCATCGAC 9293
199 TTCAAGGAGGNCGGCAACATCCTGGGGCACAANCTGGAGTNCNGCTACAT 248
9294 TTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAATACAT 9343
249 CAGNCACAACTGTATATCACCGCGCAGAGAAGAACGGCATCAAGG 298

Cyan → Stop
499 CACTCTCGGCATGGACGAGCTGNACAAGTCCGGACTCTAATAATACGTA 548
17240 CACTCTCGGCATGGACGAGCTGTACAAGTCCGGACTCTAATAATAGCGTA 17298
549 CGACGCGTCCGGATCATAATCANCATACCACATTTGTANAGGTTTTACT 598
17299 cgacgcgt.CGGATCATAATCAGCCATAACCACATTTGTAGAGGTTTTACT 17347
599 TGCTTTAAAAAACCTCCACACCTCCCCCTGAACCTGAAACATAAAATGA 648
17348 TGCTTTAAAAAACCTCCACACCTCCCCCTGAACCTGAAACATAAAATGA 17397
649 ATGCAATTGNTGNTGNTAACTTGTTTANTGCGGCTTATAATGGTACAAA 698
17398 ATGCAATTGTTGTTAACTTGTTTATTCAGCTTATAATGGTACAAA 17447
699 TAAAGCNATAGCATCNAAATTTCAATAAAGCATTTTTTNCNTGCA 748
17448 TAAAGCAATAGCATCACAATTTACAAATAAAGCATTTTTTCACTGCA 17497
749 TTCTAGTTGTGGTTTGCNAACTCATCAATGNATCTTATANGTTCTG 798
17498 .TTCTAGTTGTGGTTTGTCCAACTCATCAATGTATCTTATGTCATGAAG 17546

771 CCTTACCCENAATAGACCTCTGAAAGANAAGCAGGCACCTTTACATGGAGT 722
 14404 ccttaccacacatagacctctgacagagaagcaggcactttacatggagt 14453
 721 CCTGGTGGNAGAGCCATAGGCTACGGTGTAAAAGAGGCAGGGAAGTGGTG 672
 14454 cctgggtgggagagccataggtacggtgtaaaagaggcaggggaagtgggtg 14503
 671 GTGTAGAAAAGTCAGGACTTCACATAGAAGCCTAGCCACACCAGAAATG 622
 14504 gtgtaggaaggtcaggacttcacatagaagcctagccacaccagaaatg 14553
 621 ACAGACAGATCCCTCCTATCTCCCCATAAGAGTTTGAGTCGAAGCACCG 572
 14554 acagacagatccctcctatctccccataagagtttgagTCGAAGCACCG 14603
 571 GT 570
 14604 GT 14605

198 & 199 both have the
 extra ~600 bp Age-Age
 in 5' and have ATGs (≥ 5)

Inverted 198 seq as 198 seq, & translated:-

241 CAGAAATGACGACAGATCCCTCCTATCTCCCCATAAGAGTTTGAGTCGAAGCACCGGT 300
 GTCTTTACTCTCTGTCTAGGAGGATAGAGGGGTTATCTCAAACCTCAGCTTCGTGGCCA
 Q K * A M T I C P R O H K S L S R S T G -
 R N D R Q I P P I S P I R V * V E A P V -
 E M T D R S L L S P P * E F E S K H R C -
 301 GCTTCGACTGACTAAGTACGCTTCCTGCTGGCATCTGCTGACCTCCCCAGTGGC 360
 CGAAGCTGACTGATTGATCTTCCACTACCCGCTAGGACACTGGGGAGGGGTCACGG
 A S T D * L E A * W V A S L * P L P S A -
 L R L T N * K L D G W H P C D P S P V P -
 F D * L T R S L (M) G G I P V T P P Q C L -
 361 TCTCCTGGCCCTGGAAGTTGCCACTCCAGTGGCCACCAGCCTTTGCTTAATAAAATTAAG 420
 AGAGGACCGGGACCTTCAACGGTGAGGTACGGGTGGTCCGAACAGGATATTTTAATTC
 S P G P G S C H S S A H Q P C P N K I K -
 L L A L E V A T P V P T S L V L I K L S -
 S W P W K L P L Q C P P A L S * N * V -
 421 TTGCATCATTTTGTCTGAGTAGTGTCTCTATAATATATGGGGTGGAGGGGGTGGT 480
 AACGTAGTAAACAGACTGATCCACAGGAAGATATTATAATACCCACCTCCCCACCA
 L H H F V * G V L L * Y Y G V E G G G -
 C I I L S * V S F Y N I (M) G W R G V V -
 A S F C I R C P S I I L W G G G G W Y -
 481 ATGAGCAAGGGCAAGTTGGGAAGACAACCTGTAGGGCCTGCGGGGTCTATTGGGAACC 540
 TACCTCGTTCCTCCGTTAACCCTCTGTTGGACATCCCGAGCGCCAGATAACCTTGG
 (M) E Q G A S W E D N L * G L R G L L G T -
 W S K G * V G K T T C R A C G V Y W E P -
 G A R E L L G R Q P V G P A G S I G N Q -
 541 AAGCTGGAATGCTGGCAATCTTGGCTCACTGCAATCTCCGCTCCTGSGTTCAAGC 600
 TTGACCTCAGTCAACCGTGTAGAACCAGTGTAGAGGCGGAGGACCAAGTTGG
 K L E C S G T I L A H C N L R L L G S S -
 S W S A V A Q S W L T A I S S W V Q A -
 A G V Q W H N L G S L Q S P P R G F K R -
 601 GATTCTCCTGCCTCAGCTCCCGAGTTGTGGGATCCAGGATGATACAGGCTCAG 660
 CTAAGAGGACGGAGTCGGAGGGCTCAACAACCTAAGGTCCTACGTACTGGTCCGAGTC
 D S P A S A S R V V G I P G M H D Q A Q -
 I L L P Q P P E L L G F Q A C M T R L S -
 F S C L S L P S C W D S R H A * P G S A -
 661 CTAATTTTGTGTTTTTGTGTAGAGAGGGGTTTACCATATTGCCAGGCTGGTCTCCAA 720

Not start in 5' but
 the extra sequence
 as cloning vector.

Sun. Sep. 9th

M'21 BW-GN.AC (contd.)

Some small doubt remains concerning the AMHC border with (Cyan)... a possible extra Age 300bp Age fragment... sequence from Cyan back in red direction. Primer difficult because GN & C (Cyan) have much sequence in common. Try:

198 seq	M'29 Red Cyan primer	Comments:	198 seq	199 seq	M'21 BMHCHGNAMHC (Cyan)
		Original	TTT	CTC	ACC ATG GTG
		Determined	GGG	A	← Cyan
		Length	19		
		% GC	58%		

04:53 AM
04:59 AM
1.5%
3/10 AMHC
Mon Sep. 10th

App
Water
PCR

1+15 AMHC Not/Age frag^t ← TH4-94 5.5kb

4.5+20 AMHC Not/Age frag^t with 1st Age digest to look for 500bp Age Age.

PCR
Water
Dye

Conclusion: There was an extra Age 300bp Age piece in the frag^t. Cut again. Repurify & reassemble.

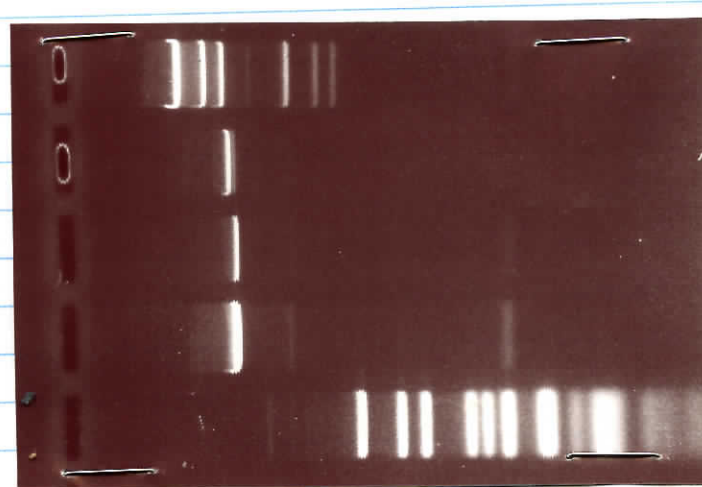
70W ←

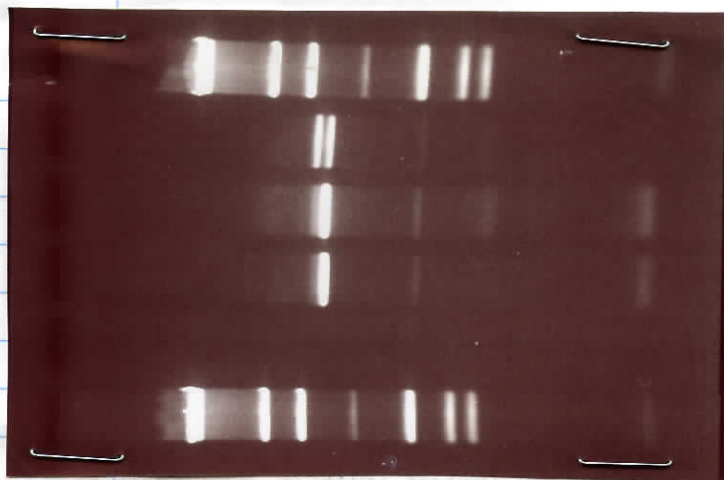
✓ (19) tcgccaccatggtgagcaa 1
14605 TCGCCACCATGGTGAGCAA 14623

os.test check: 3!
/afs/isis.unc.edu/home/n/o/nobuyo/1
25000

ASSEMBLE September 6, 2021 18
laps: 0 Quality: 150 Ratio: 10.0

✓ (15) caccatggtgagcaa 1
8090 CACCATGGTGAGCAA 8104





① Conclusion: as expected.
 Proceed to remake
 B for GN. AC.

pected.

Tues. Sep. 11th

Bhr GN.A.C (contd.)

World Trade Center -
towers destroyed by
high-jacked A/C.

T.H. digested rest of the Not - Age Age fragment
& will attempt without purification. This will
yield somewhat, but acceptably so with
help of Bgal. to eliminate BS contamination.

Meanwhile, rather messed up samples: -

① ~~0.12:05~~ ~~0.1:05~~ ~~0.1:05~~
0.8% max RNAse 3/10

(H) →

Cause & Effect.

Age
nt

Age

Pie Age

Post Age

3+13

New Age digest

W

Ag

W

Dye

rescued & muddled by mix up & overheat.

)

)

)

)

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 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100

M133
20 Bhr G N. AC

← x

←

Scrap will try ~~of~~ 16

~~2020/01/01 - 2020/01/01~~

← (20) → Degraded with overnite with Pine I
Grow up from ~~stems~~ retransform.

On 10:05a
Off 11:35a
0.80 Hx4
3/10 RNAse
max

Fri. Sep 14

M'33 contains
same pink *

Dye
AGMM

Blr GN. AC new candidates

M'21 starting

- 1) T.H, S.H. & J.C. isolate 24 white candidates using
- 2) the AgeI cut frag^t ligation. Digest overnight
- with Not.

23) JC4-91 mini digest/ Call them M'33

24)

M'29 Not

AGMM

AGMM

Confirm running back from
Red Cyan into AMHC PRO

(Reversed)
200. seq
M'33/20

M'33/20

M'29 Red Cyan

Confirm running back from
Red Mlu through Not I
& pcr A to (cyan).

(Reversed)
201. seq
M'33/20

M'33/20

K'17 Red Mlu

Confirm HGN
running into
AMHC PRO.
also sees pcr A of (cyan)
~ 300bp

202. seq

M'33/20

M'21 Blue HGN BSI

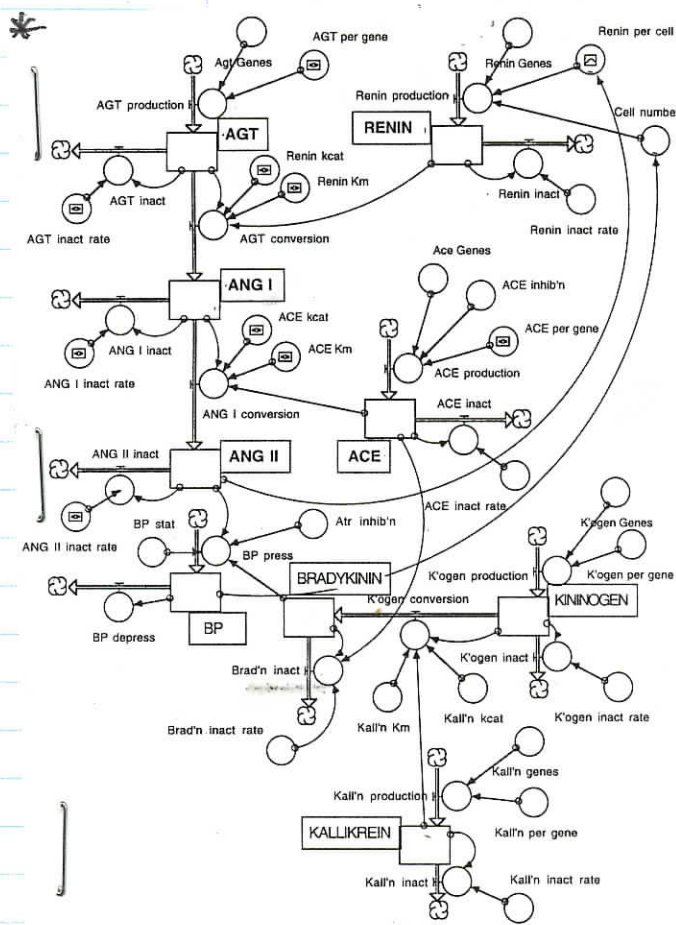
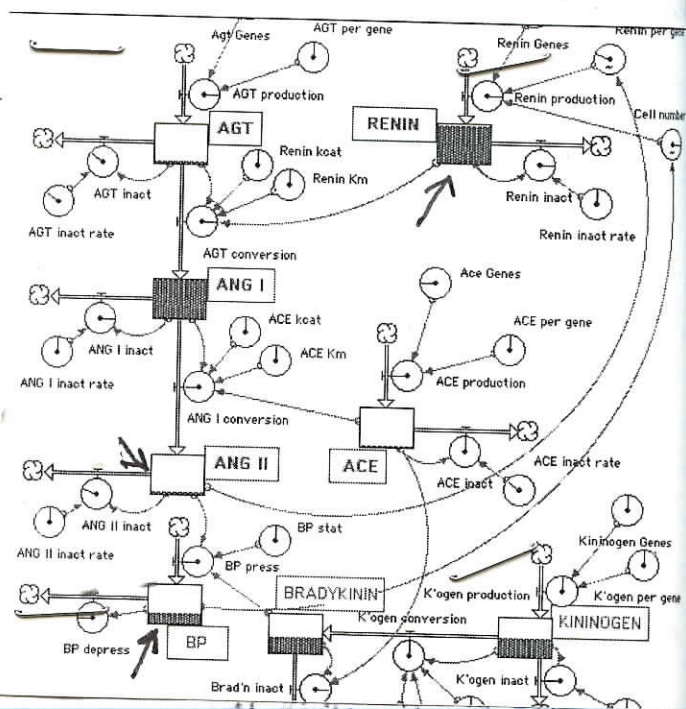
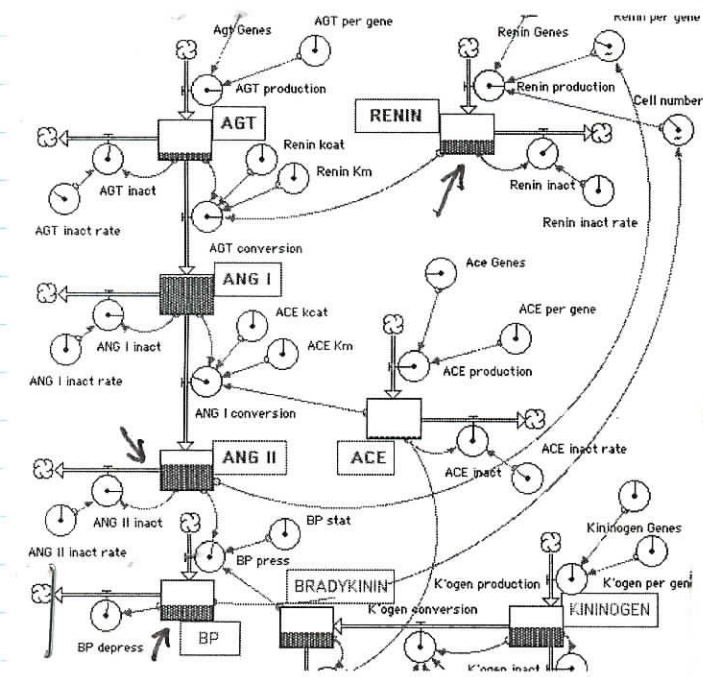
All check against
Blr GN. AC & AMHC PRO

203. seq
(Reversed)

M'33/17

M'29 Red Cyan

Rev 203 still to be checked ✓. As expected, it is perfect
match to cloning vector from 89 to 474 (or more). From 541
to 599 it matched some of pink region. Forget it!!!



BP		
BRADYKININ	17,700	39
39	2.96	156.68
1.19	5.52	152.74
1.16	9.70	148.84
1.17	15.61	146.20
1.24	22.46	140.66
1.26	28.76	127.34
1.25	33.33	115.59
1.21	36.36	101.28
1.04	38.09	86.27
0.79	39.02	74.55
0.51	39.50	67.63
0.28	39.75	63.89
0.15	39.87	61.96
0.08	39.94	
0.04		

Sun. Sep. 16 & Mon. Sep. 17th

Return to STELLA (see also M'39)

Added bradykinin machinery.

(A) Tested ^{Renin} No feedback (as old model)

(B) Tested Renin feedback via Renin per gene (old model)

→ (C) Tested Renin feedback via Renin Cell # (new test)

(D) Tested Renin feedback via Cell # and per gene.

→ (C) was very successful with same adjustments to BP stat etc. It gave ~60X ↑ in cell # and almost exactly equal ↑ in renin. BP was nicely controlled Max 157 mmHg → Min. 62 mmHg. Ang I rose & Ang II was preserved and bradykinin ↑ w/ ↓ ACE.

Tues. Sep 18th

Modified program to allow ACE inhibition as a discrete function; ditto AT₁ receptor blocking.

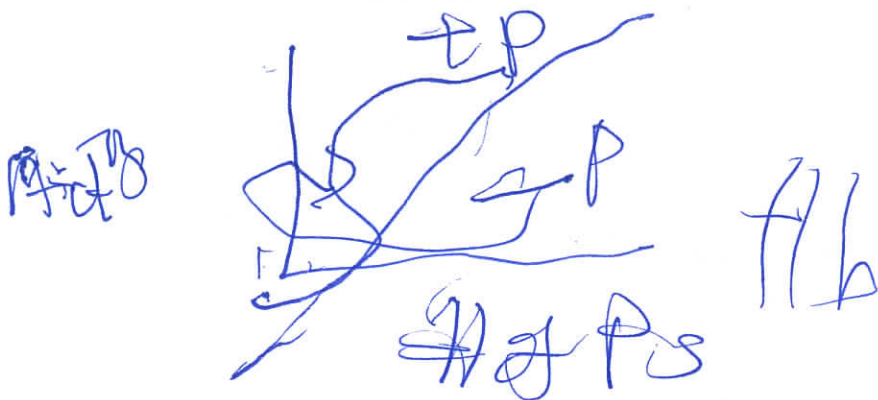
Can nicely see the extra effect due to bradykinin w/ ACE inhibitor. AT₁ is faster → more direct, as expected.

Wed. Sep. 19th

* Titled up programs 09/19/01 AT₁ and 09/19/01 ACE that demonstrate the two inhibitions. Slight differences in display in the two versions but nothing in the running. Inhibition follows a $1, \frac{1}{2}, \frac{1}{4}, \frac{1}{8}, \frac{1}{16}$ etc. activities for 14 steps from 100% to 0.01%

Plan to add Blood Volume. NPRA. Cardiac Output. Heart Rate & later kidney functions.

Sun - Mon Oct. 8th



Mon. Oct 8th

The above (!) is meant to show that ~~two~~ three things are happening:

a) phosphoryl⁻ is $\uparrow$ by stress via phosphorylating receptors/transducers.

b) dephos⁻ $\uparrow$ in same proportion as the # of phosphates/molecule

c) the auto phosphoryl⁻ has a sigmoid curve, like Hb, so that it ~~exceeds~~ exceeds exogenous phos⁻ only at a certain threshold & then causes the flip. Query: does a Hb molecule behave all or none or are they progressive? This may be known.

d) Flipping back may not be possible without actual infusion of phosphate but modelling should allow this to be tested.

Tri. Sep 28th to Oct 8th

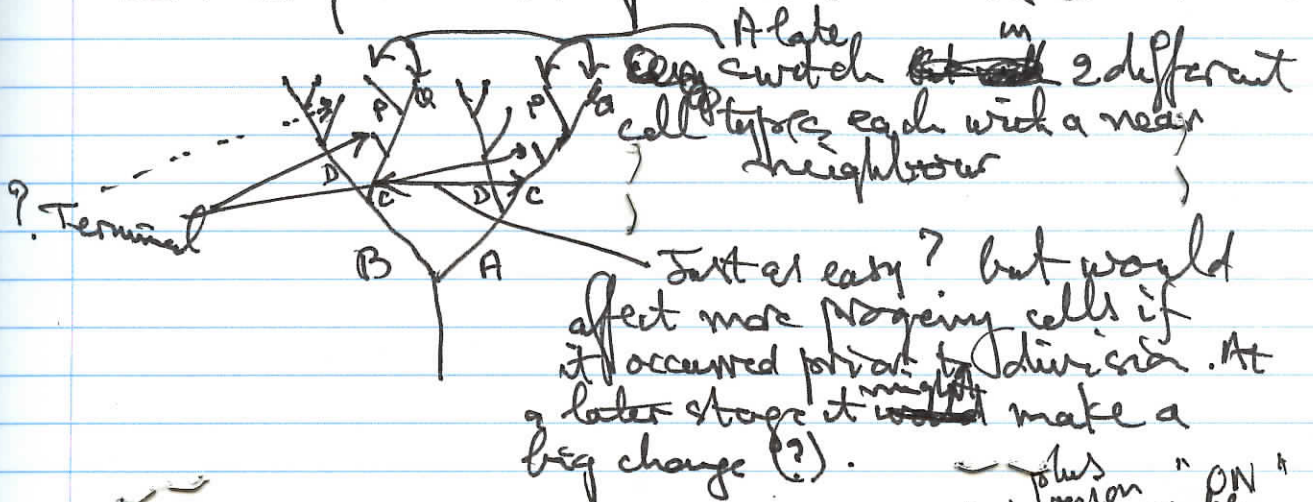
37

Program Switching

The autocatalysis threshold can be determined by protein evolution. $\therefore$ tight genetic linkage is assured i.e. crossover freq. comparable to mutation rate.

of genes of this type can be quite small.

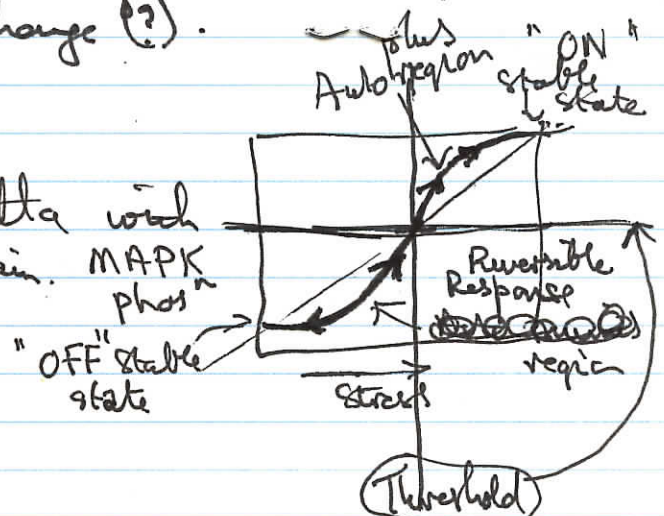
Switching between types will be odd to look at:



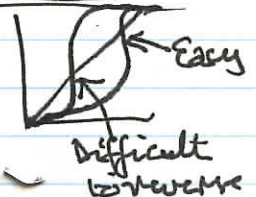
Mon. Oct 1st

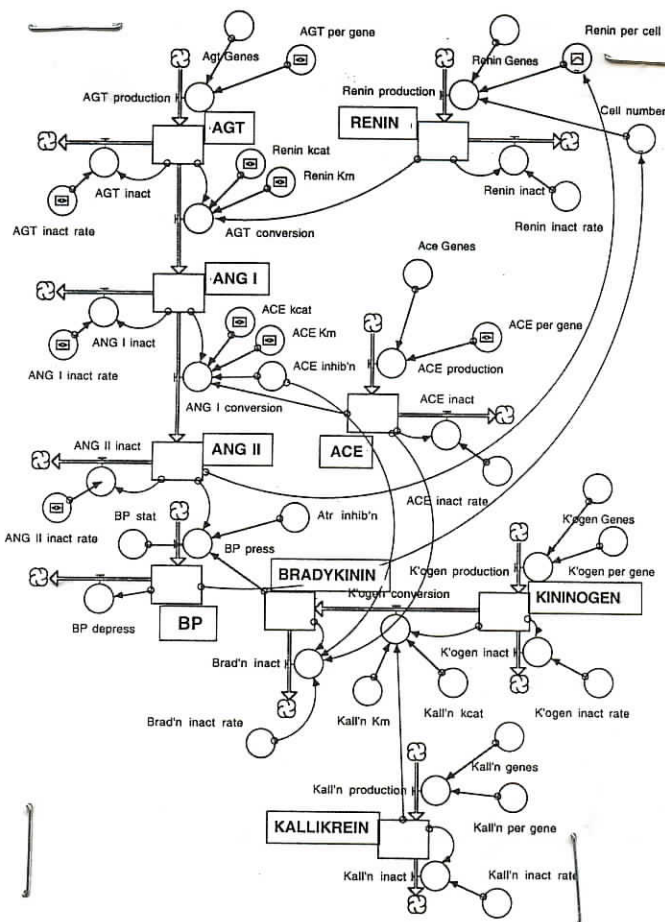
Can prob. model an Stetta with
? Read the key paper again. MAPK Phosⁿ

De phosphorylation



1) The signal(s) causing the initial flop can return to normal without flop in systems where reversibility is hard to achieve. Perhaps crossover point is determining





[INITIALIZATION EQUATIONS]

INIT AGT = 0

INIT Renin = 0

Renin_kcat = 0.1

Renin_Km = 200

AGT_per_gene = 10

Agt_Genes = 2

AGT_production = AGT_per_gene * Agt_Genes

AGT_conversion = (AGT * Renin / Renin_kcat) / (AGT + Renin_Km)

AGT_inact_rate = 0.2

AGT_inact = AGT * AGT_inact_rate

Renin_inact_rate = 0.2

Renin_inact = Renin * Renin_inact_rate

Renin_Genes = 2

INIT ANG_I = 0

Renin_per_cell = GRAPH(ANG_I)

(0.00, 1.00), (0.0033, 1.00), (0.187, 1.00), (0.25, 1.00), (0.333, 1.00), (0.417, 1.00), (0.5, 1.00), (0.583, 1.00), (0.667, 1.00), (0.75, 1.00), (0.833, 1.00), (0.917, 1.00), (1.00, 1.00)

INIT BP = 80

Cell_number = GRAPH(BP)

(0.00, 8000), (15.0, 4000), (30.0, 2000), (45.0, 1000), (60.0, 500), (75.0, 250), (90.0, 125), (105, 64.0), (120, 32.0), (135, 16.0), (150, 8.00)

Renin_production = Renin_Genes * Renin_per_cell * Cell_number

Renin_inact = Renin * Renin_inact_rate

INIT ANG_II = 0

ACE_kcat = 20

INIT ACE = 0

ACE_Km = 1000

ACE_inhib'n = 0

ANG_I_conversion = ANG_I * ACE_kcat * ACE / (ANG_I + ACE_Km * (2 * ACE_inhib'n))

ANG_I_inact_rate = 0.1

ANG_I_inact = ANG_I * ANG_I_inact_rate

ANG_II_inact_rate = 2

ANG_II_inact = ANG_II * ANG_II_inact_rate

ACE_inact_rate = 2

ACE_inact = ACE * ACE_inact_rate

Ace_Genes = 2

ACE_per_gene = 100

ACE_production = Ace_Genes * ACE_per_gene

ACE_inact = ACE * ACE_inact_rate

Atr_inhib'n = 0

BP_stat = 90

INIT BRADYKININ = 33

BP_press = ANG_II * 50 / (2 * (Atr_inhib'n)) + BP_stat * BRADYKININ * 0.75

BP_depress = BP * 1

Brady'n_inact_rate = 0.2

Brady'n_inact = Brady'n * Brady'n_inact_rate

INIT KININOGEN = 100

Kall'n_kcat = 1

INIT KALLIKREIN = 100

Kall'n_Km = 1

K'ogen_Genes = 2

K'ogen_per_gene = 100

K'ogen_production = K'ogen_Genes * K'ogen_per_gene

K'ogen_conversion = KININOGEN * Kall'n_kcat * KALLIKREIN / (KININOGEN + Kall'n_Km + 8)

Brady'n_inact = ACE * BRADYKININ * 0.0125 / (2 * (ACE_inhib'n)) + BRADYKININ * Brady'n_inact_rate

K'ogen_inact_rate = 2

K'ogen_inact = KININOGEN * K'ogen_inact_rate

Kall'n_inact_rate = 2

Kall'n_inact = Kall'n * Kall'n_inact_rate

Kall'n_Genes = 2

Kall'n_per_gene = 100

Kall'n_production = Kall'n_Genes * Kall'n_per_gene

Kall'n_inact = KALLIKREIN * Kall'n_inact_rate

[RUNTIME EQUATIONS]

AGT(t) = AGT(t - dt) + (AGT_production - AGT_conversion - AGT_inact) * dt

Renin(t) = Renin(t - dt) + (Renin_production - Renin_inact) * dt

ANG_I(t) = ANG_I(t - dt) + (ANG_I_conversion - ANG_I_inact) * dt

BP(t) = BP(t - dt) + (BP_press - BP_depress) * dt

ANG_II(t) = ANG_II(t - dt) + (AGT_conversion - ANG_II_inact) * dt

ACE(t) = ACE(t - dt) + (ACE_production - ACE_inact) * dt

BRADYKININ(t) = BRADYKININ(t - dt) + (K'ogen_conversion - Brady'n_inact) * dt

KININOGEN(t) = KININOGEN(t - dt) + (K'ogen_production - K'ogen_conversion - K'ogen_inact) * dt

KALLIKREIN(t) = KALLIKREIN(t - dt) + (Kall'n_production - Kall'n_inact) * dt

AGT_production = AGT_per_gene * Agt_Genes

AGT_conversion = (AGT * Renin / Renin_kcat) / (AGT + Renin_Km)

AGT_inact = AGT * AGT_inact_rate

Renin_per_cell = GRAPH(ANG_I)

(0.00, 1.00), (0.0033, 1.00), (0.187, 1.00), (0.25, 1.00), (0.333, 1.00), (0.417, 1.00), (0.5, 1.00), (0.583, 1.00), (0.667, 1.00), (0.75, 1.00), (0.833, 1.00), (0.917, 1.00), (1.00, 1.00)

Cell_number = GRAPH(BP)

(0.00, 8000), (15.0, 4000), (30.0, 2000), (45.0, 1000), (60.0, 500), (75.0, 250), (90.0, 125), (105, 64.0), (120, 32.0), (135, 16.0), (150, 8.00)

Renin_production = Renin_Genes * Renin_per_cell * Cell_number

Renin_inact = Renin * Renin_inact_rate

ANG_I_conversion = ANG_I * ACE_kcat * ACE / (ANG_I + ACE_Km * (2 * ACE_inhib'n))

ANG_I_inact = ANG_I * ANG_I_inact_rate

ANG_II_inact = ANG_II * ANG_II_inact_rate

ACE_production = Ace_Genes * ACE_per_gene

ACE_inact = ACE * ACE_inact_rate

BP_press = ANG_II * 50 / (2 * (Atr_inhib'n)) + BP_stat * BRADYKININ * 0.75

BP_depress = BP * 1

K'ogen_production = K'ogen_Genes * K'ogen_per_gene

K'ogen_conversion = KININOGEN * Kall'n_kcat * KALLIKREIN / (KININOGEN + Kall'n_Km + 8)

Brady'n_inact = ACE * BRADYKININ * 0.0125 / (2 * (ACE_inhib'n)) + BRADYKININ * Brady'n_inact_rate

K'ogen_inact = KININOGEN * K'ogen_inact_rate

Kall'n_production = Kall'n_Genes * Kall'n_per_gene

Kall'n_inact = KALLIKREIN * Kall'n_inact_rate

Wed. Oct. 3rd

Stella again!

10/03/01 {ACE Inhib
ACE Inhib

The M34* model is incorrect because ACE inhibⁿ is acting at the gene level. In fact it acts at the ACE level — in two places: ANGI conversion and Brdⁿ inactivation.

Rewriting equations with ACE decreasing by $1/2$ ACE inactivation (where ACE inactⁿ can be 0, 1, 2, ..., 13)

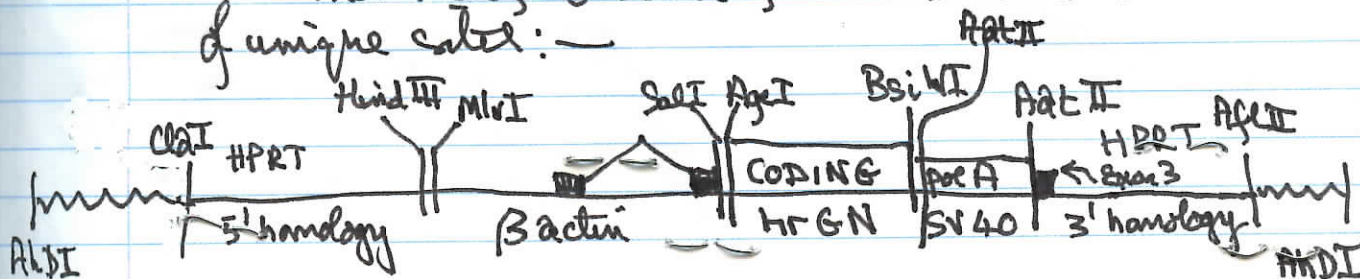
gives exactly the same result but is now logically correct.

Can add Renin to if reqd. ~~or add Renin to the model~~
Also can try to make a male model and a female model based on the data from Kim.

Sat. Oct. 6th

New vectors

The hrGN exon 3 made here will go into wild type 129 ES cells, → probably C57BL/6 cells, with TG selection. It can be used as PROM. test, CODE test, POLA. test because of unique label: —



M'41 Blue Exon 3

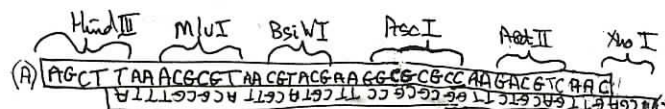
CAAAATTTG CCAATCTAGTAGGC

M'41 Red Exon 3

ATCTCC TTCATGACA TCTCGAG

New HPRT exon 3 vector

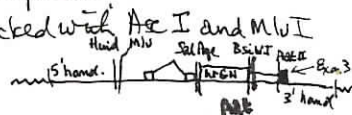
- Step ① take M'22 HPRT exon 3
 & cut with HindIII and XhoI
- Step ② Replace the HindIII — XhoI fragment with linker: —

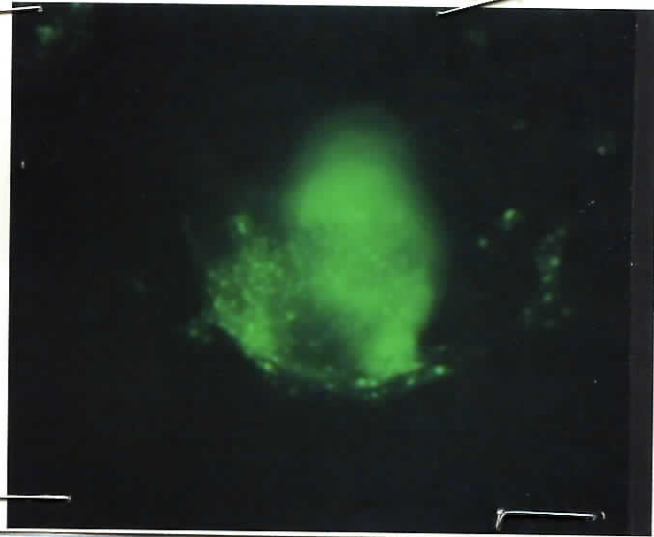
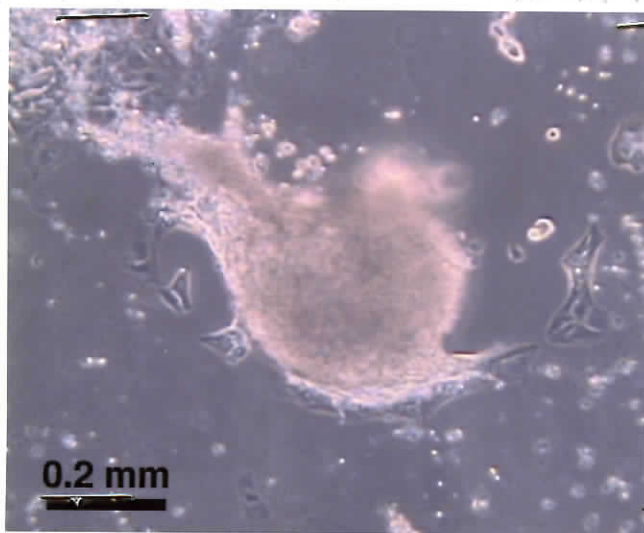
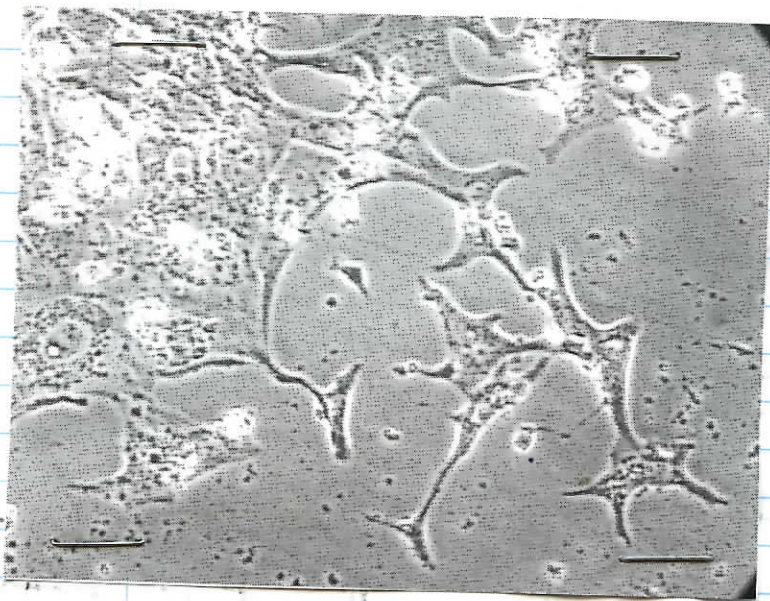


This makes M'41 HPRT exon 3 vector Made by T.H. 4-111 10/15/01

New hrGN exon 3 (targeting sequence for PROM test CODE test POLA test for use in wild type ES cells)

- Step ③ Exon 3 from Dominic's version of hrGN with MluI
 a 5.5 kb MluI ^{beta-actin} hrGN ^{SV40} MluI fragment
 (Dominic may have this already)
- Step ④ Cut HPRT exon 3 vector with MluI and AgeI and insert this MluI fragment
 Orientation can be checked with AgeI and MluI
- Product is hrGN exon 3





f
c

~

1

Computer
BGBP

Dec. 3, 2001

Sun. Oct. 14th

BMHC PURO

After 7-8 days in puro, the only surviving cells are beating, single nucleated cardiac myocytes. **AND THEY ARE STABLE!**

Caution: Scipio reports that $\xrightarrow{? \text{ PURO} \text{ hGN}}$ (in ES cells) is fine but that $\xrightarrow{? \text{ PURO} \text{ hGN}}$

gives some green.
May need to invert

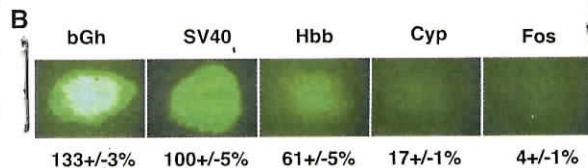
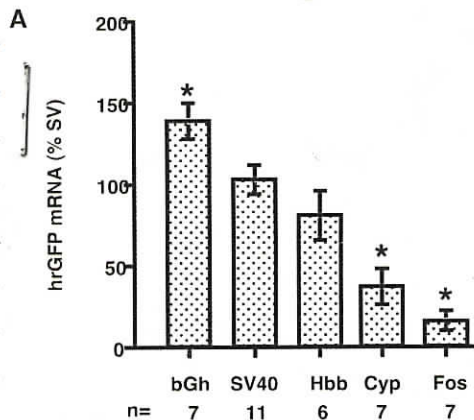
Neo in $\xleftarrow{N} A \xrightarrow{AG}$
M'23

Thurs. Nov 22nd (Thanksgiving day)

BMHC hGN. BMHC PURO

4 days of Puromycin selection on ES cells targeted with M'3 BGBP ~~blue~~ ~~only~~ ^{give} only green beating cells surviving!

(Masao)
Nice data
with 3'UTR
constructs



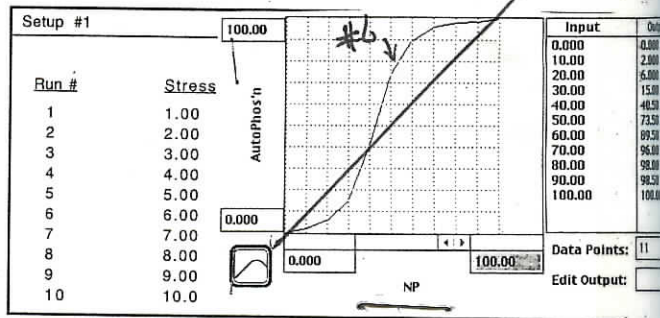
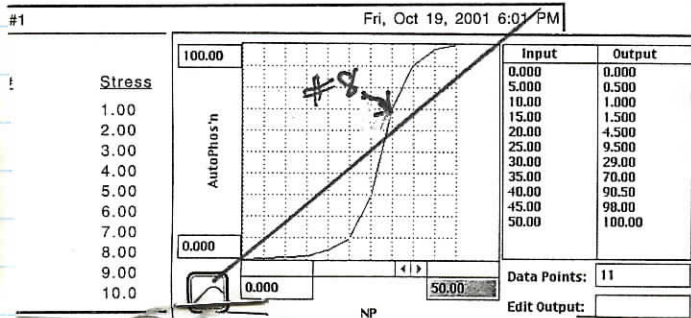
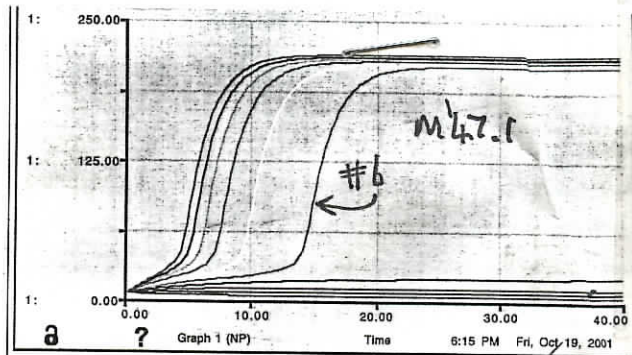
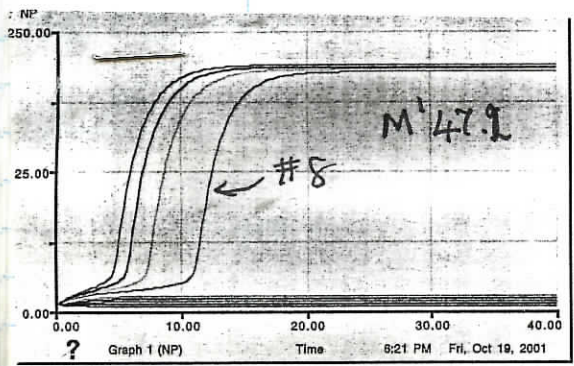
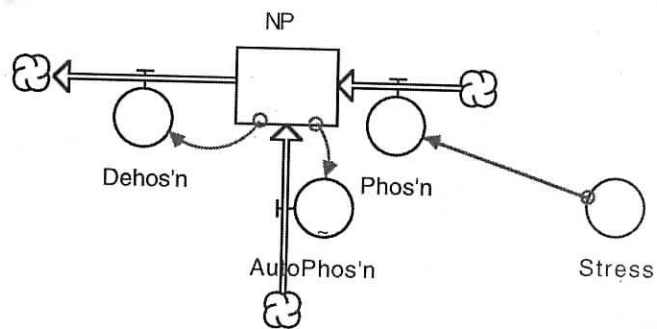
~~Tues~~
Mon - Oct 15th 16th

45

Multiple Cell Types from ES cells

Call from Arthur Sande wanting to consider
a consortium of academics to investigate ~30,000
of his mutants with (self-found?) megabuck funding.
Thought came to me that one might use
ES cell differentiation to diversify, but recognizable,
end states, characterize them by expression
analysis (including cardiac myopathy) and
then see if any of the mutants affect distal
end points — a dominant screen, but without
the need to make animals. The nuclear phenotype
of hr GN ES cell "differentiated" is very characteristic.
Could try a vital DNA stain (e.g. Hoechst 33342)
to use instead.

Interestingly, Joe tells me (Wed.) that he
is trying to identify the same cell types in
hr GN. SV40 p1A versus hr GN. bGH p1A
to compare brightness. The same thought
from different points of view.



47
Fri. Oct 19th

Bingo!!!

First significant attempt at Phosⁿ + DePhosⁿ + AutoPhosⁿ under stress worked. Can adjust the flip point by shape of auto curve.

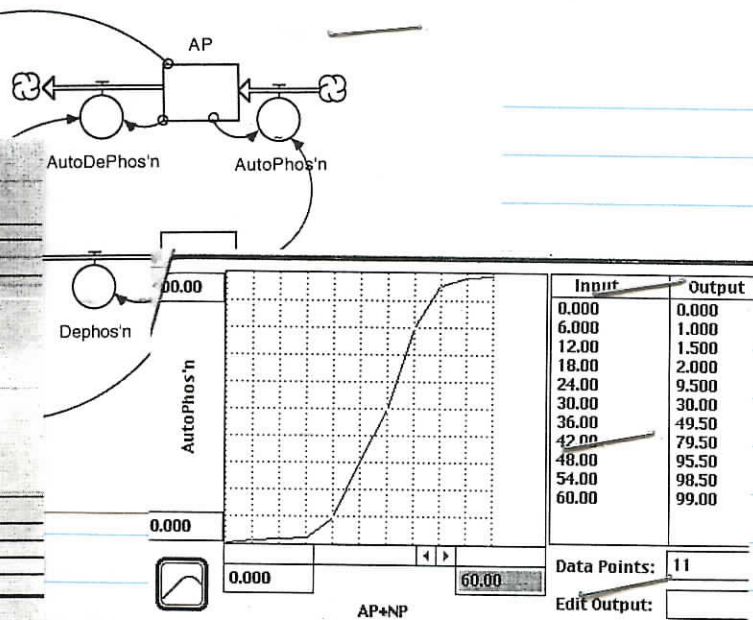
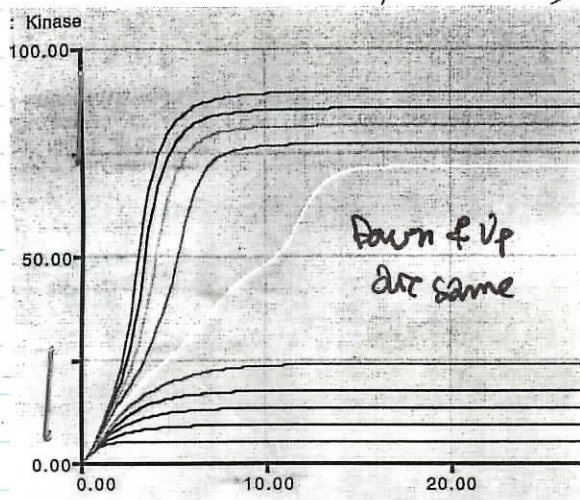
But it doesn't show hysteresis a reversal but the reversal is not a true reversal. Try again with all settings at 40 cycles.

Input	Output
0.000	0.000
0.00	2.000
0.00	5.000
0.00	15.00
0.00	40.50
0.00	73.50
0.00	89.50
0.00	96.00
0.00	98.00
0.00	98.50
100.00	100.00

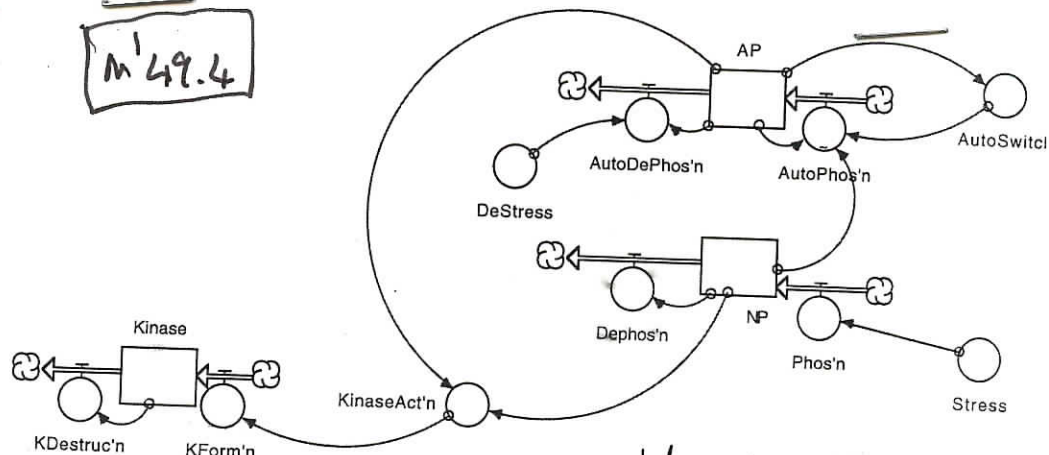
Data Points: 11

Plot Output:

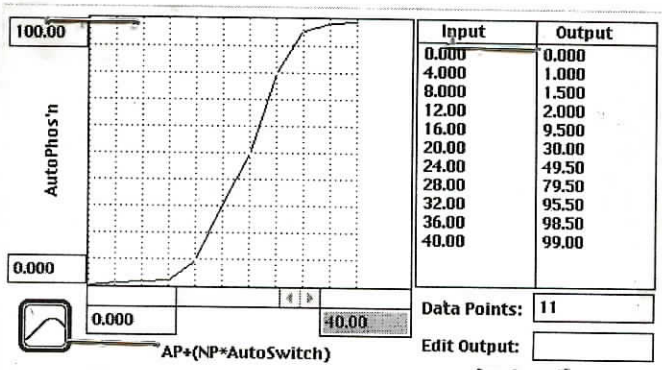
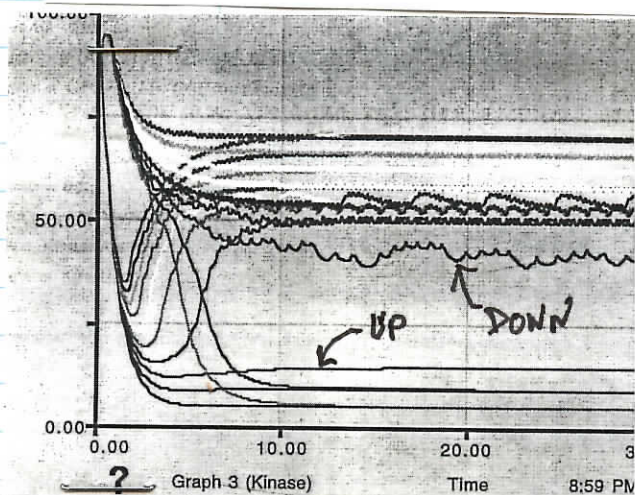
M'49.1



M'49.4



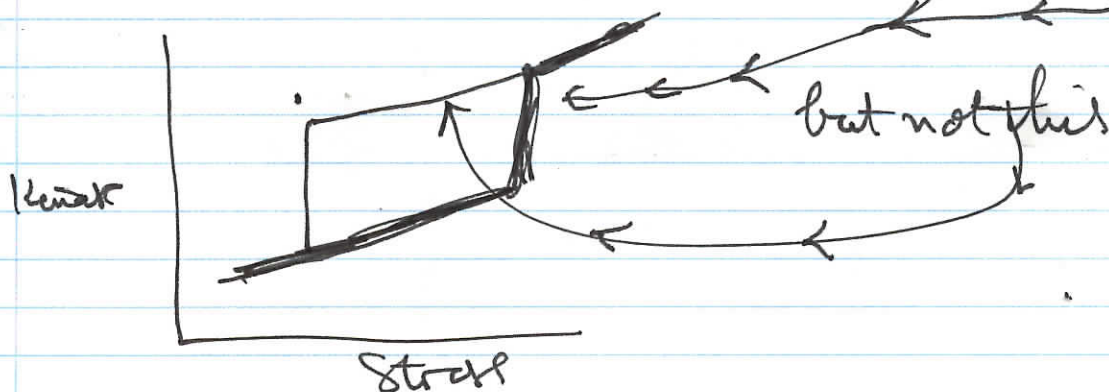
Hypertension!



Mon Oct 22nd

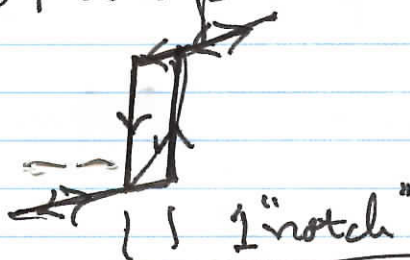
Pho / Dephos. 11 - Renamed M'49.1

Greatly improved model with Kinase Activity & separation of autophosⁿ from direct stress-induced phosⁿ. However, it still does not latch or revert on a new track. i.e. I have chis



Evening

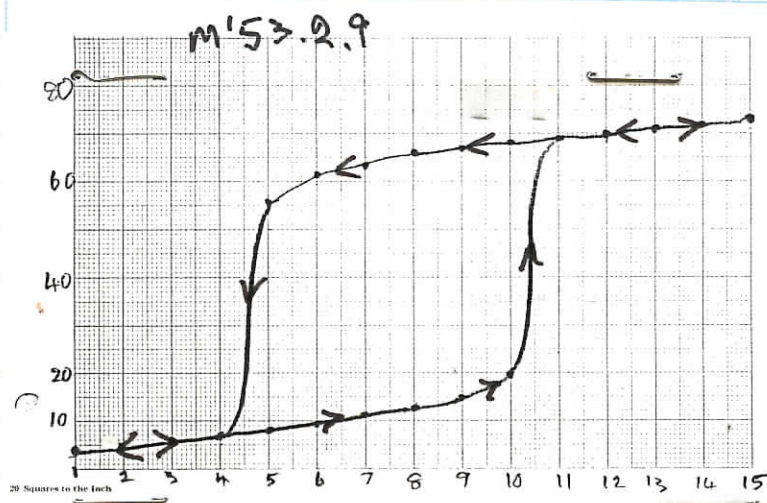
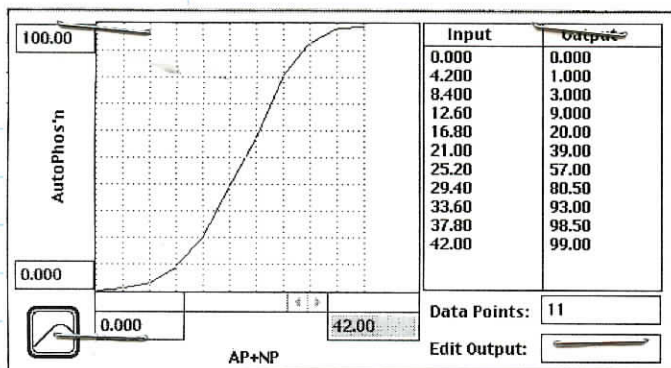
M'49.4 By switching off the stress-induced rate of autophosphorylation when autophosⁿ exceeds a threshold (i.e. has switched), the system can show hysteresis



Tues. Oct 23rd

M'49.1 shows hysteresis after all!
Forget the switch.

5 pm 2 "notches" |||



Tues./Wed Oct 23/24

51

Careful runs with M'49.5

Changing the span of Auto

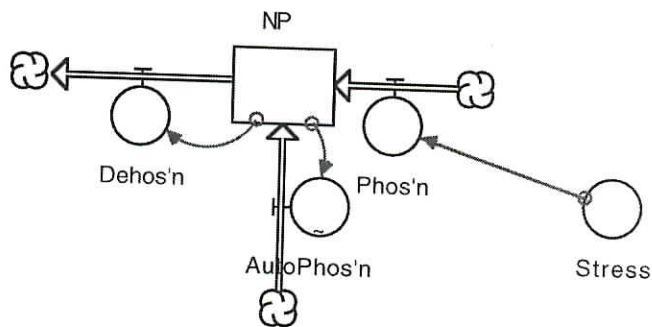
from 100 to 20

shows:

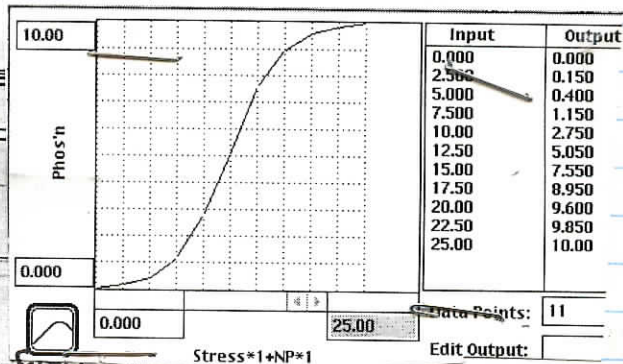
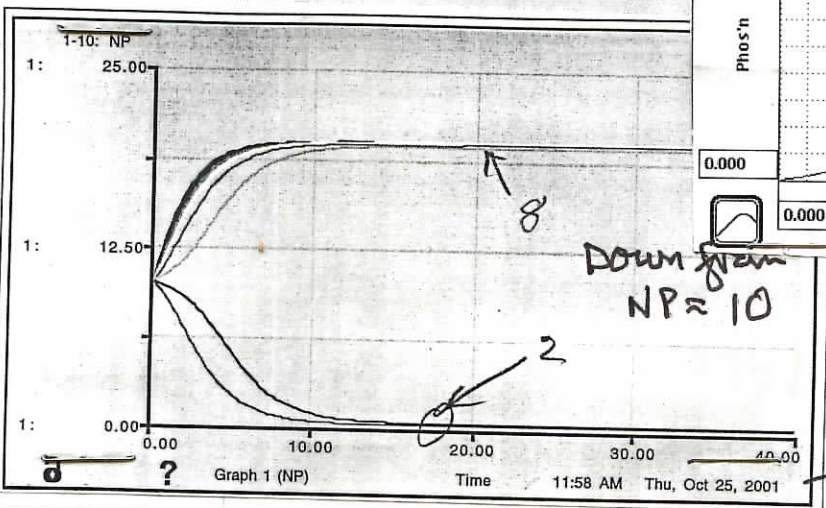
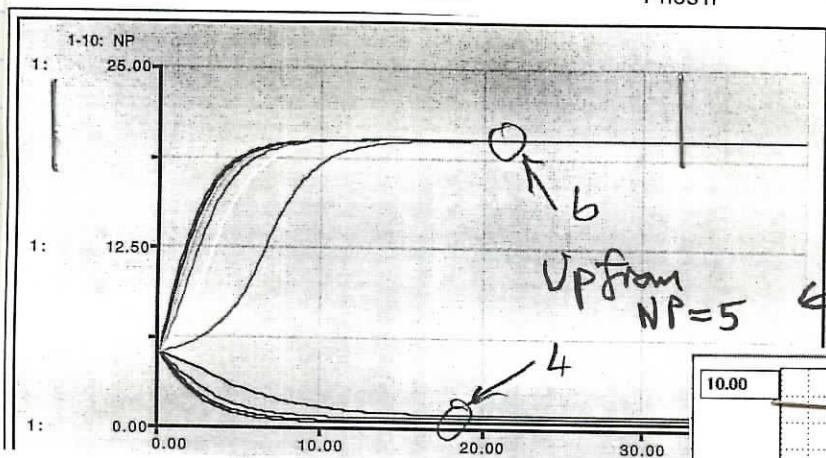
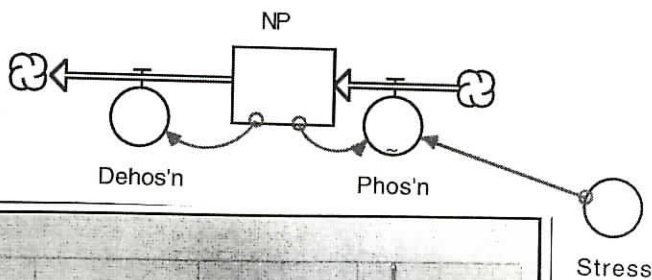


- 100 - No switching, kinase low
- 90 - $\uparrow$ and $\downarrow$ differ but still no switch
- 80 - Switch but no hysteresis
- 70 - 1 notch hysteresis
- 60 - 1 notch hysteresis
- 50 - 1 notch hysteresis
- 45 - 1 notch hysteresis, v. symmetrical
- 44 - 2 notches hysteresis
- 43 - 2 notches hysteresis, v. symmetrical
- 42 - 2 notches hysteresis
- 41 - 2 notches hysteresis
- 40 - 2 notches hysteresis
- 39 - hatched up
- 38 - hatched up
- 35 - hatched up
- 30 - hatched up
- 20 - No switches, kinase high.

Changing the steepness of the [Auto Phos.] vs. [AP+NP] curve affects result, but by careful "tuning" a flatter slope of 5 units (flatter) versus 4 units (old standard) can give excellent hysteresis. By adjusting the sensitivity range, the plots can be made quite satisfactory.



Pho/De phos 3.2



Also Pho/De phos m'53.1 Up

Down from NP=10

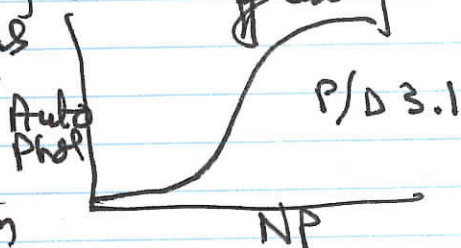
Also Pho/De phos m'53.1 Down

Thurs. Oct. 25th

Attempt to make Pho/Dephos 3
give hysteresis

3.2

Can easily adjust the flip point with the usual tuning but reverse is always latched high. Doubt if it is logically poss. When curve has X-axis of "NP", not "AP + NP". ? need to keep track of the flip rate separately



Not really true. By slight change to include Auto Phos as a function of AP + NP. Gives 2 notches of hysteresis!

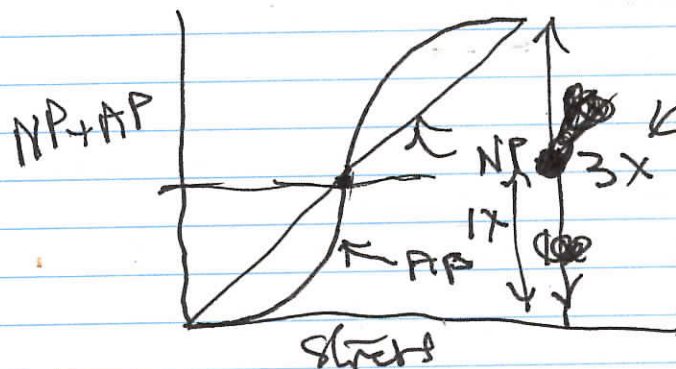
Pho/Dephos M'53.1. (Down
Up)

Fri. Oct 26th

look at effect of ratio of Auto Phos to Stress Phos for latching, hysteresis etc.

Simple scheme: M'53.1. Down/Up were 10 each

Complex Scheme: M'53.2.9 were 10 for NP and 100 for AP



OK for Up.
What about Down?

1. Down

Sat Oct. 27 - Thurs. Nov 1st

Cont. modelling

Along series of "tuning" shows that the number of "notches" is easy to adjust & that the shape of the curve is not very critical
e.g.



Fri. Nov 2nd

Approach to reality

Review by Hubbard & Till (Ann. Rev. Biochem. 2000, 69:373-398) is debated in physical properties of the Receptor Tyrosine Kinases and the NonReceptor Tyrosine Kinases (NRTK).

Src is example

The key elements in NRTKs appear to be a tyrosine (e.g. Y416) in the activation loop for positive regulation & a tyrosine (e.g. Y527) in the carboxy-terminal region that acts as a negative regulator.

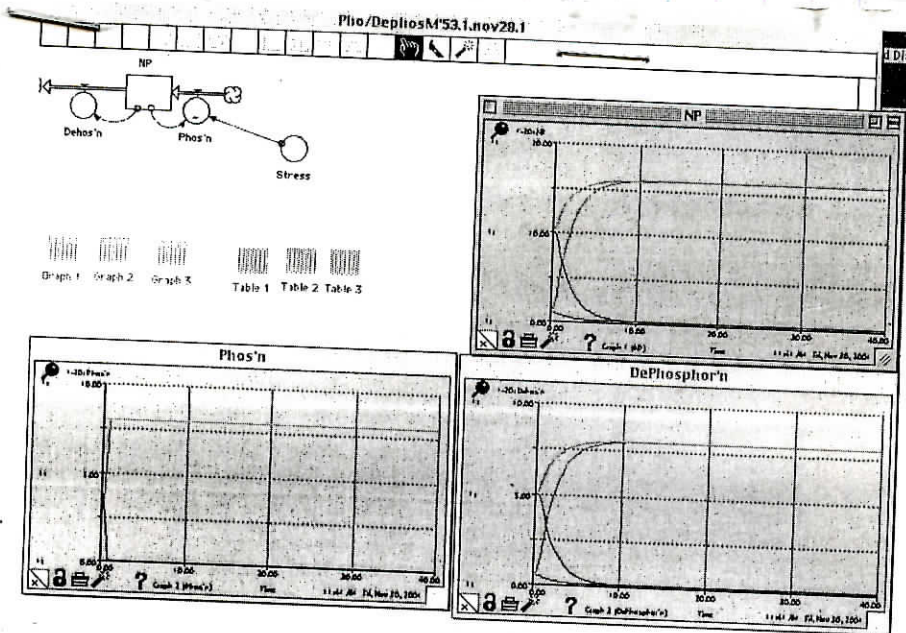
"~~Autophosphorylation~~ of Y416 is reqd. for maximal kinase activity & "merely stabilizes the active conformation of the loop". ligands can expose the Y416 for autophosphorylation.

Repression is not by occlusion of active site. "Instead, intramolecular interactions stabilize the misalignment of residues important for catalytic activity and also... hinder productive ATP binding". Protein tyrosine phosphatases (PTP) restore act. to basal.

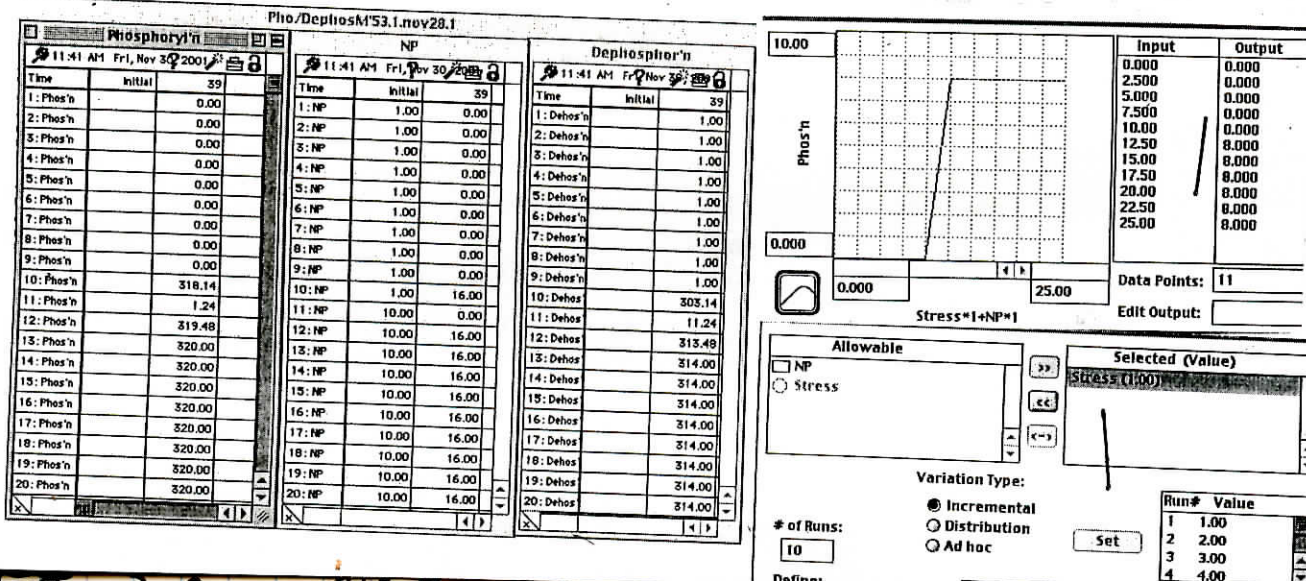
Auto Phosphor. ~~occurs~~ occurs via trans-autophosphorylation or phosphotransfer from a different NRTK. Y527 in Src is phosphorylated by the NRTK Csk. Lack of Y527 or Y527F are constitutively active. Not all NRTK have Y527.

Should be able to model that with very little complicating factor.

Tried thinking of this as a probability so that autophosphorylation is a function of $NP \times AP$. First test of a modified form of M'53.1. Down gives 4 notches!! Need to put in concn $[Kinase] \times P$



- (INITIALIZATION EQUATIONS)
- ☐ INIT NP = 10
 - ☐ Stress = 1
 - ☒ Phos'n = GRAPH(Stress*1+NP*1)
(0.00, 0.00), (2.50, 0.00), (5.00, 0.00), (7.50, 0.00), (10.0, 0.00), (12.5, 8.00), (15.0, 8.00), (17.5, 8.00), (20.0, 8.00), (22.5, 8.00), (25.0, 8.00)
 - ☒ Dehos'n = NP*0.5
- (RUNTIME EQUATIONS)
- ☐ NP(t) = NP(t - dt) + (Phos'n - Dehos'n) * dt
 - ☒ Phos'n = GRAPH(Stress*1+NP*1)
(0.00, 0.00), (2.50, 0.00), (5.00, 0.00), (7.50, 0.00), (10.0, 0.00), (12.5, 8.00), (15.0, 8.00), (17.5, 8.00), (20.0, 8.00), (22.5, 8.00), (25.0, 8.00)
 - ☒ Dehos'n = NP*0.5



From Phosphoryl in in 19.4 (s) until occurs is As the large injected in a time we would conc'n) - constraints P start. P either up or because of used as for (≡ NP*23) which the stresses (C) test. N

Pho/DephosM'53.1.nov28.1
Sample 1E
give up OK
step 10
Fri. Nov 2 10:1

Pho/DephosM'53.1.nov28.1
sample P
system up
the direction
calcs than be
turn.

More

Tues. Nov. 27th

Grant sent off 57
Probably the last ever?

More models

From ~~provisional~~ inspection of the break points in M'49.4 (see M'48) it appears that the switch occurs when the slope of autophosⁿ exceeds the linear increase due to stress.
This suggested that autophosphorylation that depends on dimerization of partially phosphorylated enzyme would also flip because of dependence on (concⁿ).² Test this with simple model with constraint that total protein should be constant. PO-3 Nov 23.1 ... 6. Flips OK either up or down but cannot handle PO=0 or down because of use of PO as a multiplier. No notches obtained in first trials. As expected on PO-3 Nov 24.1 trials ($\equiv$ Nov 23.4), the flip point is concⁿ-dependent - the higher the concⁿ, the sooner the flip with same stresses (0.00 - 0.2)

Wed. Nov. 28th

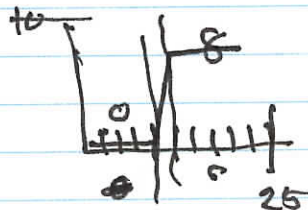
Testing PO-3 Nov 23.4 as modified to Nov 28.1 }
show PO=34, P2=1 $\approx$ PO=1, P2=34.
The system up from 1 and down from 10 shows 4 notches.
In both directions the switch occurs when Phosphatation is greater than Dephosⁿ on up, and when P is less than Dephosⁿ on down.

Thurs. Nov 29th

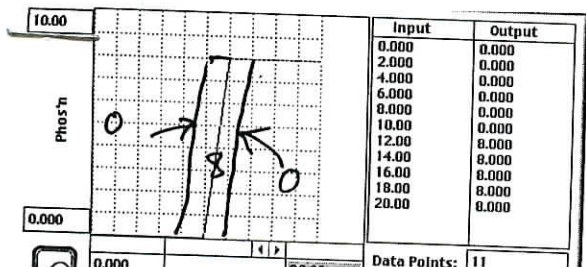
Pho/Dephos M'53.1 Nov 28.1 with very steep (one square) switch gives 8 notches i.e. max^m possible.

Fri. Nov 30th

Yet simple IF/THEN will give up OK but not down at step 10 when ≥ 10.1



(over) →



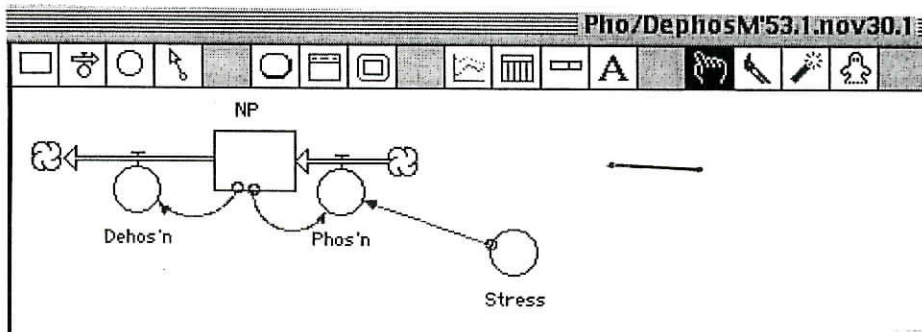
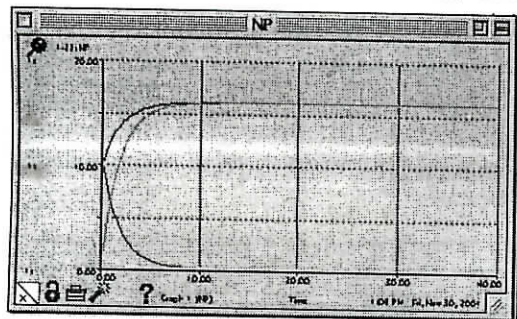
8 notches

Note

Stress 1-11 on there

Phosphoryl'n			NP			Dephosphor'n		
Time	Initial	39	Time	Initial	39	Time	Initial	39
1: Phos'n		0.00	1: NP	1.00	0.00	1: Dehos'n		1.00
2: Phos'n		0.00	2: NP	1.00	0.00	2: Dehos'n		1.00
3: Phos'n		0.00	3: NP	1.00	0.00	3: Dehos'n		1.00
4: Phos'n		0.00	4: NP	1.00	0.00	4: Dehos'n		1.00
5: Phos'n		0.00	5: NP	1.00	0.00	5: Dehos'n		1.00
6: Phos'n		0.00	6: NP	1.00	0.00	6: Dehos'n		1.00
7: Phos'n		0.00	7: NP	1.00	0.00	7: Dehos'n		1.00
8: Phos'n		298.66	8: NP	1.00	16.00	8: Dehos'n		283.66
9: Phos'n		310.98	9: NP	1.00	16.00	9: Dehos'n		295.98
10: Phos'n		315.27	10: NP	1.00	16.00	10: Dehos'n		300.27
11: Phos'n		317.43	11: NP	1.00	16.00	11: Dehos'n		302.43
12: Phos'n		2.33	12: NP	10.00	0.00	12: Dehos'n		12.33
13: Phos'n		6.01	13: NP	10.00	0.00	13: Dehos'n		16.01
14: Phos'n		200.00	14: NP	10.00	10.00	14: Dehos'n		200.00
15: Phos'n		319.11	15: NP	10.00	16.00	15: Dehos'n		312.11

Doesn't change since equal
NP=10 Phos'n=5 Dehos'n=5



- { INITIALIZATION EQUATIONS }
- ☐ INIT NP = 1
 - ☐ Stress = 1
 - ☐ Phos'n = IF((Stress*1+NP*1)>11)THEN(8)ELSE(0)
 - ☐ Dehos'n = NP*0.5

- { RUNTIME EQUATIONS }
- ☐ NP(t) = NP(t - dt) + (Phos'n - Dehos'n) * dt
 - ☐ Phos'n = IF((Stress*1+NP*1)>11)THEN(8)ELSE(0)
 - ☐ Dehos'n = NP*0.5

Allowable

☐ NP

☐ Stress

Selected (Value)

Stress (1.00)

Variation Type:

☒ Incremental

☐ Distribution

☐ Ad hoc

of Runs:

11

Define:

Graph

Table

Start: 1.00

End: 11.0

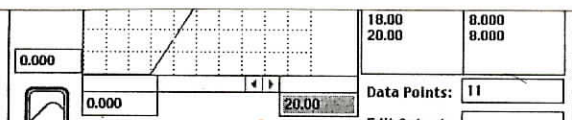
Run# Value

1	1.00
2	2.00
3	3.00
4	4.00

☒ Sensitivity On

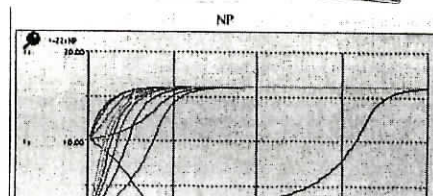
☒ Print Setups

Cancel OK



Input	Output
0.000	0.000
2.000	0.000
4.000	0.000
6.000	1.100
8.000	2.350
10.000	3.750
12.000	5.150
14.000	6.650
16.000	8.000
18.000	8.000
20.000	8.000

2 notches



Tric Nov 30th

Threshold
Sample toggle

Stress 1 thru 10

Phosphorylⁿ IF $((\text{Stress} \times 1 + \text{NP} \times 1) \geq x)$ THEN (8) ELSE (0)
Initial NP = 1 or 10.

Greater than or equal	$x = 1$	Up at all	Up at all
	$x = 2$	Up at all	Up at all
	$x = 3$	Up at 2	Up at all
	$x = 4$	Up at 3	Up at all
	$x = 5$	Up at 4	Up at all
	$x = 6$	Up at 5	Up at all
	$x = 7$	Up at 6	Up at all
	$x = 8$	$\vdots$ 7	$\vdots$
	$x = 9$	$\vdots$ 8	$\vdots$
	$x = 10$	Up at 9	Up at all
	$x = 10$	Up at 10	Up at all
	$x = 11$	Up at 10	Up at all
$x = 12$	Down at all	Up at all	

✓ X

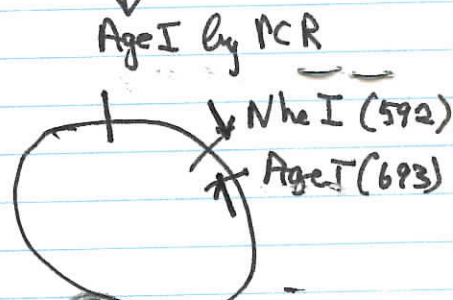
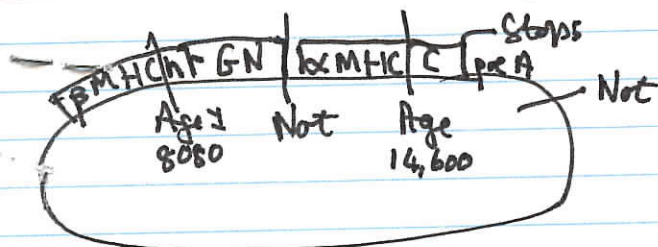
Greater than	Changing logic to $\dots \geq x \dots$	Down at all	Down at 1	X ✓
	$x = 11$	Down at all	Up at all	
Greater than	Changing stress to 1 thru (11) with $x = 11$			✓
	Gives 9 notches Up at 11 Down at 1			✓

Can't get any simpler. Nov 30.1
Changing slope is okay but ↓ notches Nov 30.2

Fri. Nov 30th

Bhr GN. ~~AC~~ A mito C

Decided to change AC (cytoplasmic) to A mito C.
Can start with M'21 Bhr GN. AC & do single
cut Age I, followed by insertion of Nhe I mito Age I
from Clontech ~~into~~ pDs Red 2-Mito



M'61 Red Red is red primer for pDs Red 2-Mito just
into Met of Red

M'61 Age ~~Red~~ Mito Blue is blue primer for pDs Red 2-Mito
5' to Mito ATG + 8 bp introdup. Bam will help orient &
is new in that region. Bam will help orient & close, to 3' end of fragment

Correct orientation will give a Bam HI fragment
of 1.0 kb (incorrect will be 1.1 kb) plus 5.6 kb
(incorrect will be 5.5 kb); no insert will be 6.5 kb

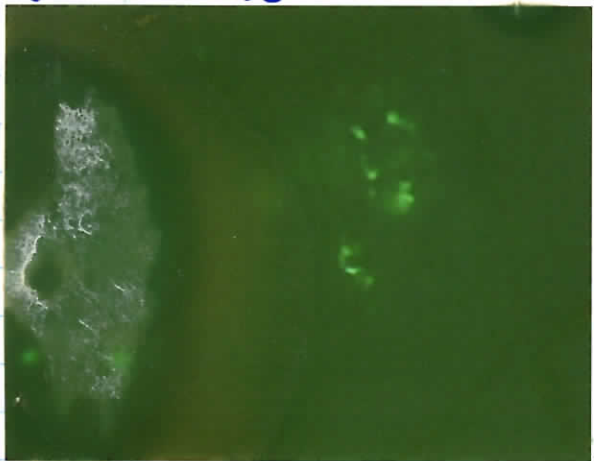
Design ~~error~~
~~ATG~~

		M'61 Red Red				
31494		GAC	GTT	CTC	GGG	GGG
20		GGC	CA			
65%						
	725	706 in Red 2-Mito				
		M'61 Age Mito Blue				
31495		AAA	AAC	CGG	TGA	TCC
29		GCT	AGC	ATG	TCC	GT
62%						

But I used it as pDs Red 1-Mito
which probably explains PCR problem.

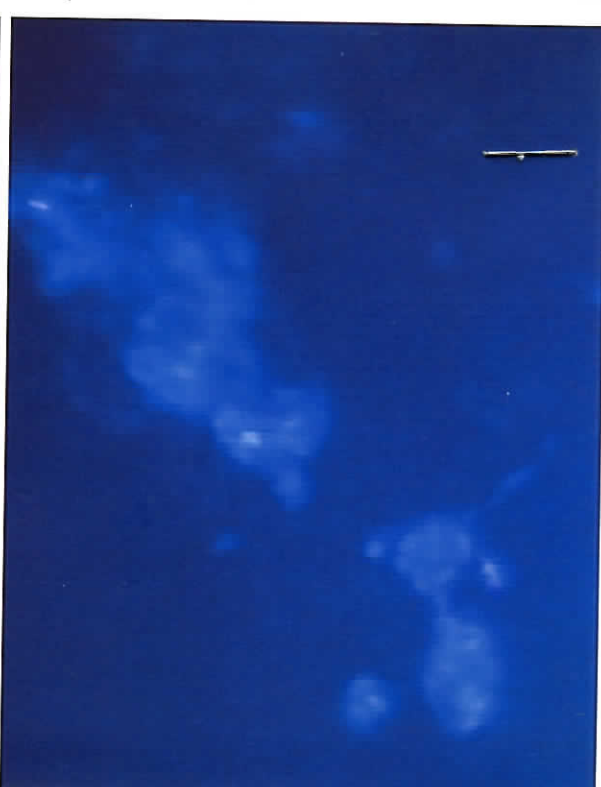
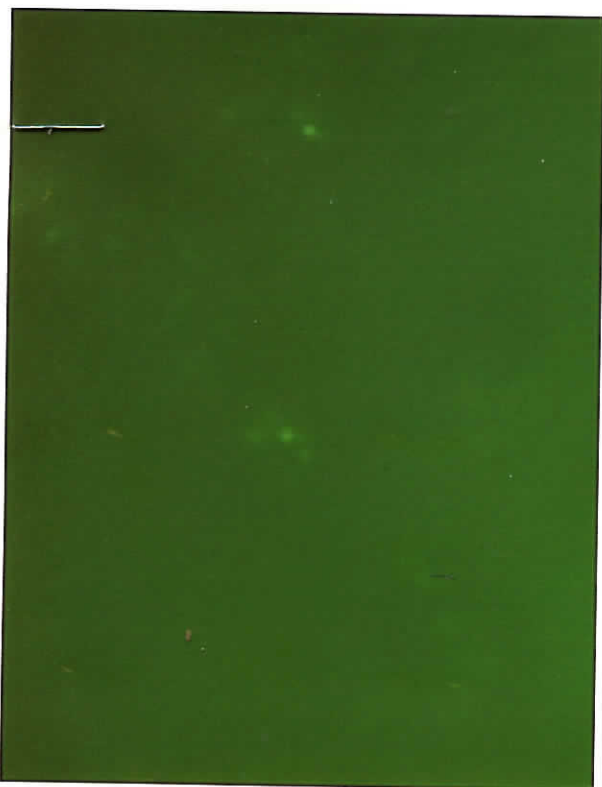
joe Nov 2.1 jpg from 1024/01

1024-5, 1024-9



joe Nov 2.2 pad

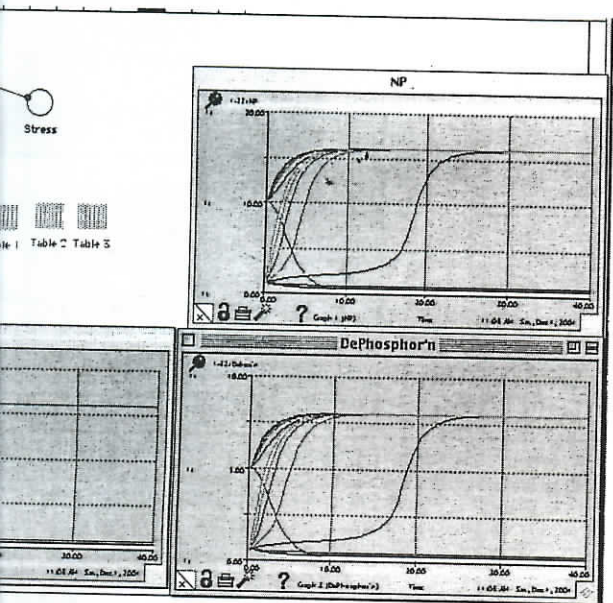
10 Brightness 35 contrast



M'33. BHCN.AC

63

Some points with β MHC h r G N α M H C C
after differentiation



Input	Output
0.000	0.000
2.000	0.000
4.000	0.150
6.000	0.400
8.000	1.050
10.00	2.800
12.00	5.950
14.00	7.400
16.00	7.850
18.00	8.000
20.00	8.000

Data Points: 11

Edit Output:

Delete Graph Cancel OK

Pho/DephosM53.1.dec1.1

Phosphoryl'n			NP			Dephosphor'n		
Time	Initial	39	Time	Initial	39	Time	Initial	39
1: Phos'n		7.54	1: NP	10.00	0.00	1: Dehos'n		17.54
2: Phos'n		316.35	2: NP	10.00	16.00	2: Dehos'n		310.35
3: Phos'n		318.69	3: NP	10.00	16.00	3: Dehos'n		312.69
4: Phos'n		319.50	4: NP	10.00	16.00	4: Dehos'n		313.50
5: Phos'n		319.78	5: NP	10.00	16.00	5: Dehos'n		313.78
6: Phos'n		319.92	6: NP	10.00	16.00	6: Dehos'n		313.92
7: Phos'n		319.98	7: NP	10.00	16.00	7: Dehos'n		313.98
8: Phos'n		320.00	8: NP	10.00	16.00	8: Dehos'n		314.00
9: Phos'n		320.00	9: NP	10.00	16.00	9: Dehos'n		314.00
10: Phos'n		320.00	10: NP	10.00	16.00	10: Dehos'n		314.00
11: Phos'n		320.00	11: NP	10.00	16.00	11: Dehos'n		314.00
12: Phos'n		0.00	12: NP	1.00	0.00	12: Dehos'n		1.00
13: Phos'n		0.18	13: NP	1.00	0.00	13: Dehos'n		1.18
14: Phos'n		3.67	14: NP	1.00	0.18	14: Dehos'n		4.50
15: Phos'n		8.20	15: NP	1.00	0.40	15: Dehos'n		8.80
16: Phos'n		14.76	16: NP	1.00	0.73	16: Dehos'n		15.02
17: Phos'n		203.34	17: NP	1.00	16.00	17: Dehos'n		188.34
18: Phos'n		299.30	18: NP	1.00	16.00	18: Dehos'n		284.30
19: Phos'n		311.55	19: NP	1.00	16.00	19: Dehos'n		296.55
20: Phos'n		315.91	20: NP	1.00	16.00	20: Dehos'n		300.91
21: Phos'n		318.09	21: NP	1.00	16.00	21: Dehos'n		303.09
22: Phos'n		319.07	22: NP	1.00	16.00	22: Dehos'n		304.07

m'58 bp left
h the
a pu

Sum
be due
to be
modification
ran a
f
Blue

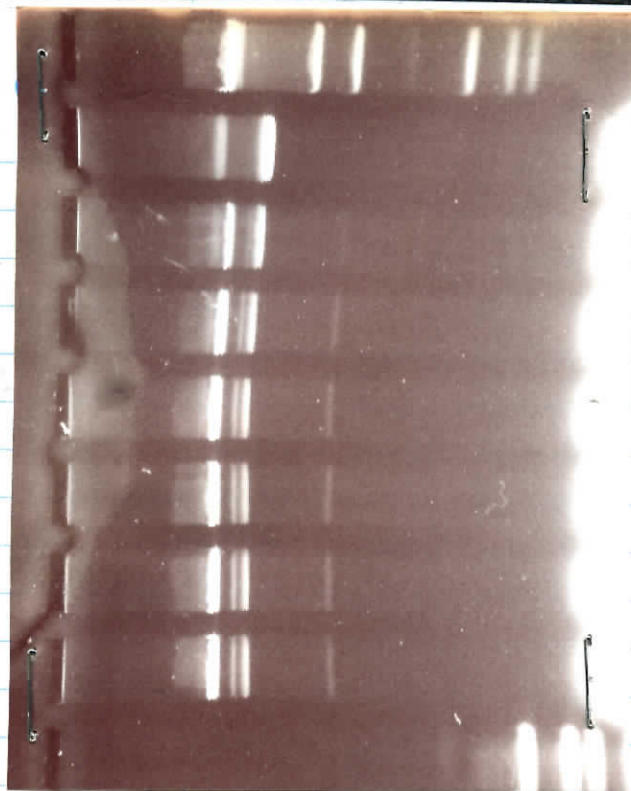
Sat. Dec. 1st

Snatches abrupt, modification

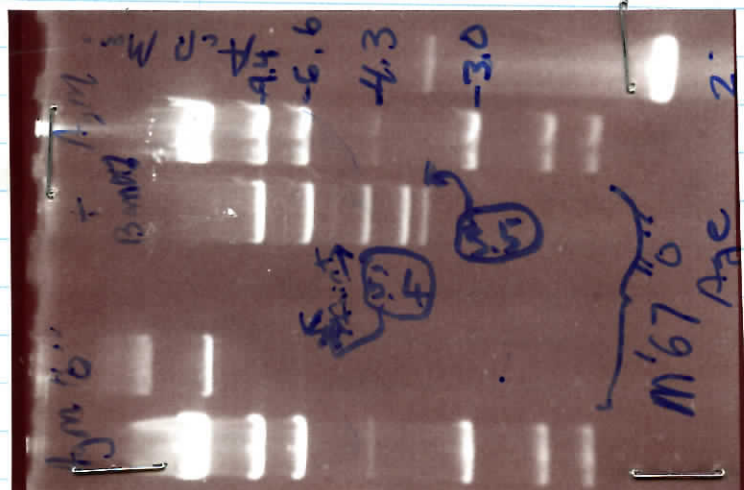
M'58 bp left 8 notches was modified to smooth the transition. Result is a gate increase 5% in NP → then a pump on ↑, and an abrupt drop on ↓.
 Phosⁿ is smooth on ↑ but abrupt on ↓
 Depthⁿ is also " " " " " " ↓.
 Better quit this work for a while.

Sun. Dec. 16th

Can use the BMHC^{Puro}. BMHC^{Neo}. AMHC^{Neo}. AMHC^{mito} Cydr
 ES cells to transfer a transgene, introduce a
 gene modification to remove a type or cer
 group from a candidate gene → check effects a
 ES in culture culture.
 i.e. check at protein
 level is possible.
 Blue ↔ Green

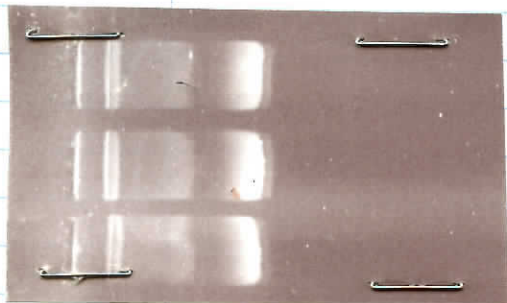


Conclusion: Good range. Don't limit because of timer. Pool 1, 2, 4, 8 & 16 for prep. gel after CIAP. It's possible that one of the two Age sites is difficult to cut in R1 results, as the time series is plateauing. Test O with another enzyme. Don't need CIAP since gel will be extracted with ϕ OH. Faulty reasoning! CIAP is needed to prevent self-closure.



J.C. 2% Ag 2x Holdings

Bad pinching problem between agarose & scaffolding. Try no agarose.



11:50am
12:46p
0.8% HX4
max

Ma. Dec. 3rd

Single cut Age M'33 Bhr GN.AC

Dye

W

AGMM

C

"0"

1

2

4

8

16

ACRMM

W

Dye

Take 20 pl of T.H. M'33 Pool #20 Jc4-91

X + 40 pl 10% RISalt

X 330 pl ddw

X warm to 37°

✓ Add 10 pl (20u) Age I lot 34, 2u/pl

Control

✓ 20 pl

60 pl samples

0, 1, 2, 4, 8, 16

Best time

Inactivate $\geq 75^\circ$ ≥ 10 min

0.8% Soap. HX4 1.5v/cm

Dye

N 1480ts

Dye

M'67 Age Pool

M'67 "0"

J.C. tested "0" with Bam HI. Clear evidence that the hr GN Age I is the preferred (unfortunately wrong) site.

Choices are to fill in or turn back & self close & then cut again with Age or 2 to go ahead & accept the ratio (~ 1:10). ① looks better.

Bam alone on Bhr GNAC should give:

(Age)	Site (Bam)
690	3.9 kb
4540	4.6 kb
9110	50 bp
9160	6.5 kb
15620	10.1 kb
(25017)	690

Diagnostic

3.5 kb

1 kb

5.4 kb

1 kb

Medium strong

Faint

Error

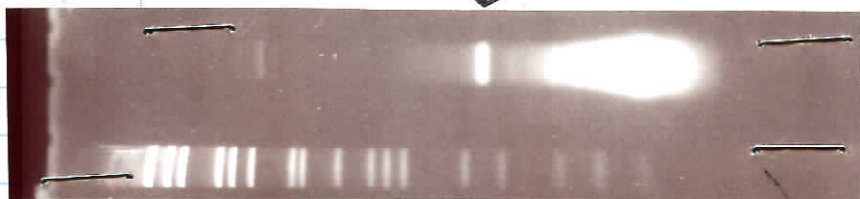
1st gel separation lost because of contaminated tube!!



①

Conclusion: The product appears to be too short (~100 bp); sh'd be (150 bp). longer washing look promising. Ppt. and run purification gel.

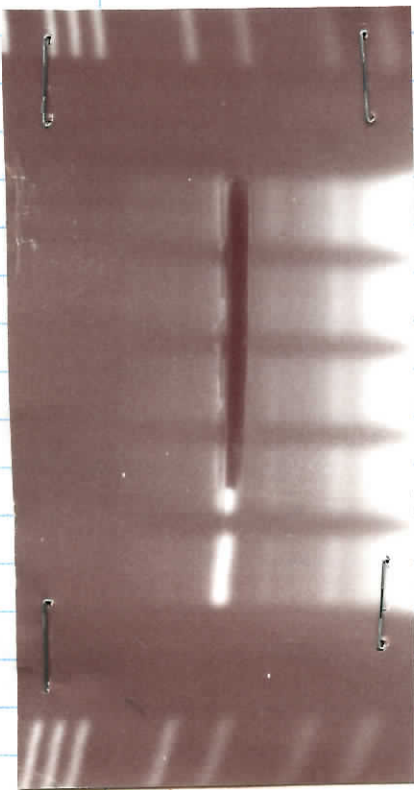
↑ 100 bp
↑ 150 bp



↑ 124
↑ 142
102
56/55
79/80

②

Repeat a M'69 PCR product show expected band. Digest with Age & reamplify. Save a little for reamp., if nec?



③

Prepare a try Age I again & reamplify with less primer.

92 µl crude + 230 µl EtOH

- back into 50 µl as M'68 mto fragt

9:30 am
on 10/1/11
①

on 10/1/11
②
11
CM

PCR for "mito"

PCR 500 pL rx with K^+ 49 pDs Red-1-MitoVektor as template. (K⁺ protocol)

$n=20$ M'69 ~~20x~~ 20x PCR buffer 25 μ l ✓
 ddw 362 μ l ✓
 M'69 ~~20x~~ DMSO 50 μ l ✓
 M'69 ~~20x~~ dNTPs 2 μ l ✓
 K'49 pDs Red 1-Mito Vector 1 μ l ✓
 M'61 Red Red primer 20 μ l ✓
 M'61 Age Mito Blue primer 20 μ l ✓
 480 μ l
 to 5 rx tubes, 90 μ l each
 Heat to 93°. Add 4 μ l regular Tag to each tube $\rightarrow$ reanne cycle $\geq 90^\circ$ 1 min
 Good start 2:50 pm 40° 7 sec 35 cycles.
 60° 8 min

290 H x 1 Saphyng
m'69 PCR Product

②

CN 2:40pm
3:35pm Hx1
2:40pm SV1

M'69 PCR/Age

③

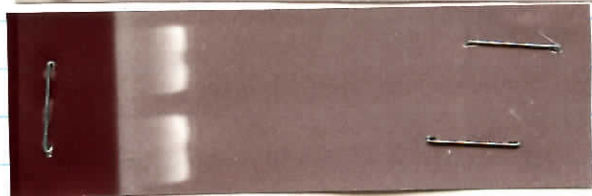
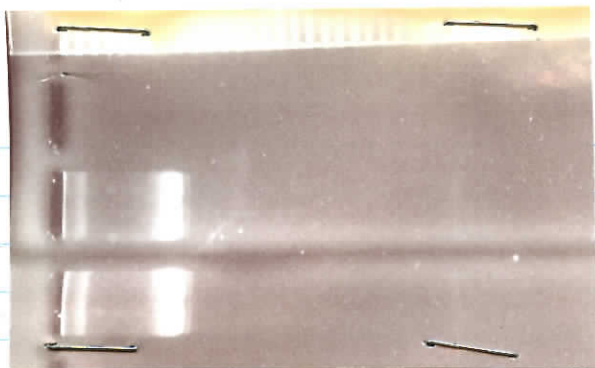
LACRMM

Nobuys out under u.v. — no sign
of Age Action.

Apt! black into 100 plas
 Thurs Dec 6th

M'69 PCR
 Product

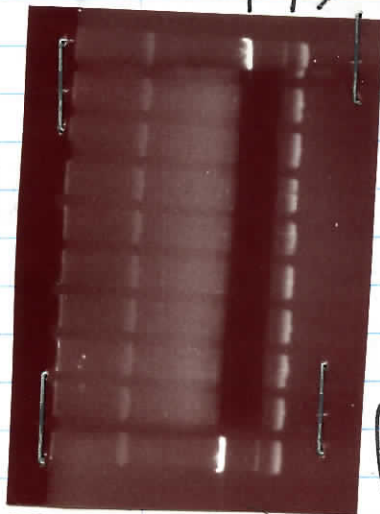
Age I on PCR Frag^t
prior to reamplification
M'69 PCR Prod.^t
10x R1 Salt
2.5 μ l (5v) Age I lanes 3 4 6
37° 10:55 to 2 pm



John says that the
Cyan Age I was the
site that gave him a problem.

Nothing recovered!
Try purifying out of agarose.

single
double cut
No cut



810mg

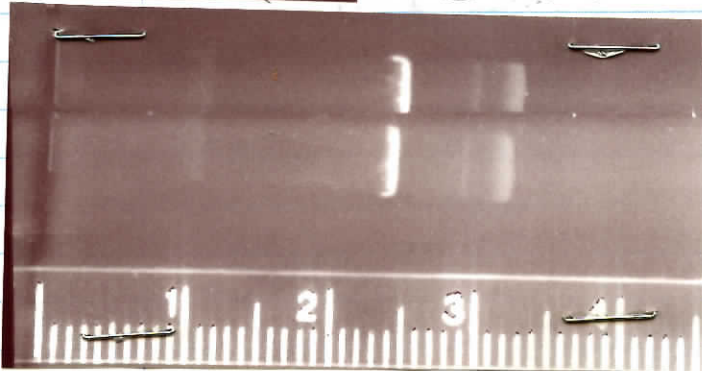
Free & thawed (+ 80 μ l 8MNH₄OH)
Several times.

Mashed & phenol
extracted. Recovered
550 μ l (promising).

Back into 50 μ l
as M'70 single cut Age

Gel on
M'72

Need to Klenow
& self-ligate,
or Sequenase



0.8% Hx4 on
max

on 12:30
off 1:30

IntP
W

ACR M M

W

Mashed up PRE

POST Klenow

POST Klenow / W

AG M M

W

off 1:30

on 12:30

off 1:30

off 1:30

off 1:30

off 1:30

off 1:30

off 1:30

off 1:30

off 1:30

off 1:30

off 1:30

off 1:30

off 1:30

Thurs. Dec. 4th

Repeat single cut Age M'33 Bhr GN.AC

Same mix as M'67 but gave 10 min total time.
Heat inact! 10 min $\geq 80^{\circ}\text{C}$

on 4:15 pm
off 9:30 am

Age 1.5 V/cm

Entirely 0.8% Sap

~ 14 slots $\leftarrow$ $N \times 4$

(slots were usable by waiting for larger set)

M'71 Age

don't! $\rightarrow$ back into 100 μl as M'71 Frag.

Thurs. Dec. 6th

~~Erra~~ Klenow fill in of M'71 Frag^t

~~Take 50 μl (50%) M'71 Frag^t~~

~~+ 2.5 μl 2.5 mM 4 dNTPs D'9~~

~~+ 2 μl Klenow Boer. Mann. lot 2003 20 μl~~

~~+ 6 μl $\phi 103$ 10X NT buffer~~

~~+ 6 μl R.T. — to —~~

~~+ 6 μl 10X p95 lig⁻ buffer~~

~~+ 2.5 μl T4 ligase~~

~~37° — to —~~

~~16 μl M'71 Post Klenow~~

~~M'71 Klenow~~

~~(100 μl final)~~

~~See T103~~

~~P127~~

~~Factor old, gel has solidified~~

Restart

- ✓ Take 50 μl (50%) M'71 Frag^t
- ✓ + 2.5 μl 2.5 mM 4 dNTPs D'9
- ✓ + 6 μl $\phi 103$ 10X NT buffer
- ✓ + 2 μl Klenow polymerase lot 2003

RT 10:40 am to 11:20 am

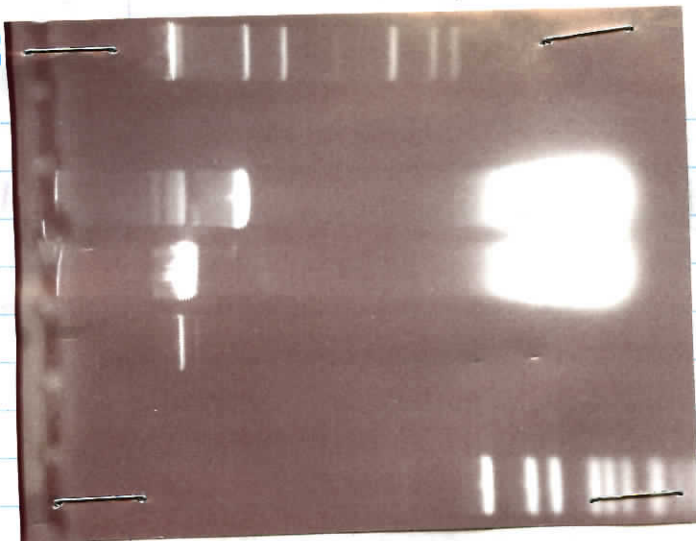
- ✓ + 5 μl 10X p95 lig⁻ buffer
- ✓ + 2.5 μl T4 ligase

37° 11:20 to 12:20 & later

M'71 Klenow/lig⁻

Change to 5' 7' separate protocol

16 μl M'71 Post Klenow



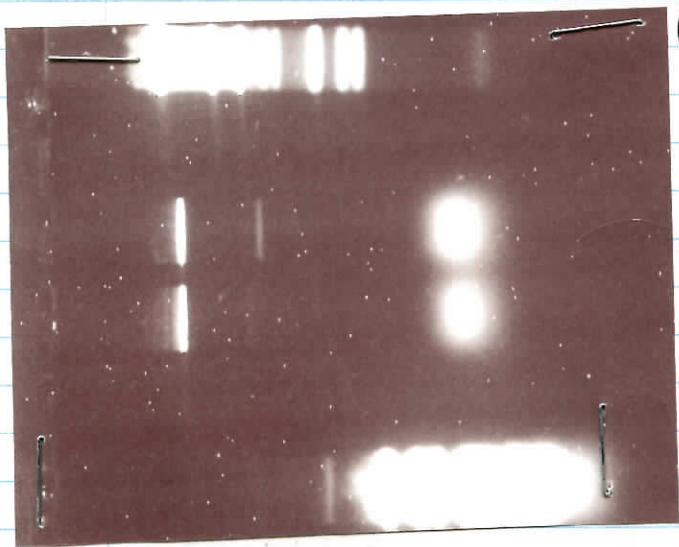
① Conclusion:
MLV cut
good; L115 is
Pero. Single
cut Age is work
pulling.

On 4:15
5:45
0.8
Hx
4x on

①
Dyr
W
Ag MM
W
M'73 P
23 Post M
Undiluted M'70
W
AER
W
Dye

BMP

Single
site



② Conclusion:
good location,
but could
↓ vector by 2x *
if repeat is nec?

On 11:17
11:47
0.8

Dye
W
Ag
W
PRE
POST
W
AER
W
Dye

M'7

(✓)
6+
M
P
2

Thurs. Dec. 6th

BMHC PURO + β Actin hr GN

Seigo needs that combination for infection into animals to find the cells from β MHC PURO. β MHC hr GN (see M'42) if they go elsewhere $\rightarrow$ shut off β MHC promoter. Can make from

M'10 BMHC PURO (L'115) with insertion of Mlu frag⁺ from Dominic's M'10 β Actin hr GN pEA M'10 construct (already isolated).

Same confusion exists in labelling of L'115. BMHC. PURO and L'119 BMHC. hr GN; it can be resolved with Mlu - L'115 has one site; - L'119 has 3 sites.

Fri. Dec. 7th

Take β pl L'115 Red 17 β MHC PURO
 6 pl 10x RI salts
 51 pl ddw
 1 pl Mlu (10u) lot-26 (10u/pl)
 37^o 3:15pm to 4pm $\rightarrow$ on to 6:00pm (frozen)
 16 pl M'73 PRE
 + 175 pl + 50 pl IM Tris pH 8
 + 2 pl CIAA (5x CIAA buffer)
 50^o 2:15pm to 2:50pm
 1x 40H/HOQ 2x CIAA
 20 pl + 2 pl Dominic's Mlu β Actin hr GN pEA Mlu
 + 2 pl 10x p95 (M'73/C75)
 + 2 pl T4 ligase L'99
 Cold room 3:10pm to 11^{am} to sat.
 6+10 M'73 PRE LIGW
 M'73 lig⁺
 6+10 M'73 POST LIG⁺

Thurs. Dec. 6th

Single site Age (contd.)

Single cut of Bhr GN.AC obtained M'70 Single cut Age

Age ↑ Main cut ↓ Age ↑ Minor cut

Need to blunt this with Klenow fill in, religate, isolate or sequence

→ react with Age I to insert Age I cut PCR frag!

This will improve discrimination of green & blue growth.

M'68 single frag!

This may be a problem.

Mon. Dec. 10th

Blunt
Bhr Age Age GN, AC

- ✓ Take 30 µl M'70 Single cut Age
- + 8.4 µl 5X Sequencer Buffer lot 82572 USB M'77
- ✓ 1.7 µl D9 2.5 mM 4 dNTPs to give 100 µM final
- ✓ 1 µl (2u) Klenow Polymerase lot 2003

R.T. 3:15 to 3:30 pm

M'77 Age blunt Age lig

+ 4.8 µl 10X P95 buffer

+ 2 µl T4 ligase

Heat inactivated ≥ 80°C 10 min

37°C 4:05 to 6:25 pm → to cold

Transformation

79 DH5α
M'77 Age blunt Age lig
6:26 to 7:06 pm
42°C 90 sec.

Plated
1XAMP 1XNZY
Rest 20.5 20/ 5/ 16/ 16
(15) (7) (9)

Tues
37°C 7:15 pm
(cont'd)
M'83

Mini picked 12:30 pm
 1XNZY 1XAMP 37°C
 plates were dry
 colonies were mostly hard to pick.
 Not true to lig.

100 µl processed to KATC & cold.

Bcl WI 4kb + 19 kb

(3)(10)(11)(13)(14)(15)(19)(20)

K Red

M'79 BAC hrgN.BP

(and more)

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

(12)
(14)
(15)
(17)to
Seigo

M'79 BAC hrgN rev. BP

(1)(2)(4)(5)(6)(7)(12)(16)(23)(24)

Bcl WI 9 kb + 1 1/2 kb

Blue

Confirm by sequencing with K'17 Blue Mlu primer

	Primer	DNA	Water
211. seq	K'17 Blue Mlu / 100 ✓ 5 µl	M'79 (12) 2 µl	✓ 13 µl

	Primer	DNA	Water
212. seq	K'17 Blue Mlu / 100 ✓ 5 µl	M'79 (15) 2 µl	✓ 13 µl

Dominic thinks it
might be a copy
problem. ∴ Sequence
M'79 (15) with
K'17 Red mlu

M'79 (15) (212. seq) vs. M'79 BAC hrgN.BP

confirm, (15) is M'79 BAC hrgN.BP

1 TTTGGTATCCCAATTACCGCTGTATTCGACTTTCAACAGCCTTTGG 50
4386 TTTGATAT.CGAATTACCGCTG.AATTCCGACTTTCAACAGCCTTTGG 4533
51 ACTCAGGCCTTGGGGACATTCAGGGGACCTTTACAGGGTAGGGGCGAG 100
4534 ACTCAGGCCTTGGGGACATTCAGGGGACCTTTACAGGG.AGGGGCGAG 1582
101 TGTGAGGCAGGAGGAGGGCTGCCCTCAGGGTACCTGAGCCTAGAGT 150
4583 TGTGAGGCAGGAGGAGGGCTGCCCTCAGGG.ACCCTGAGCCTAGAGT 4631
151 CTTCCACCCGATTTGGGGACTTGGCACTCACCAGTCTGACTCAGT 200
4632 CTTCCACCCGATTTGGGGACTTGGCACTCACCAGTCTGACTCAGT 4681
201 TTCTAGTCTGTGAATGCGCTGTCTCTCTGGAATGTTGCCCTCCCTCA 250
4682 TTCTAGTCTGTGAATGCGCTGTCTCTCTGGAATGTTGCCCTCCCTCA 4731
251 TTCAATTCATCTCTCTCACTGAGCACCTACCGTNTCAGGGTCCCTGTC 300
4732 TTCAATTCATCTCTCTCACTGAGCACCTACCGTNTCAGGGTCCCTGTC 4781
301 CTCTAGAGCTAACTCAAGTCTTGGAGGGAAGCTGTCTCAGNTICTT 350

3 TTNTCTAGTNTCCNAATTGACCGCTGCGTAGGCGAGACTACCAATT 52
4479 TTCTATATTTGATATCGAATTACCGG...TAGGCGAGCTGAATT 4523
53 AGCTNCGTAGGGCTTCGAGGACATGTATAAGTATACATTGCATGAGTT 102
4524 AGCTTCGAGG...ATCCAGACATG.ATAAG.ATACATTG.ATGAGTT 4566
103 GGACATACACACTACTAGCAATGCAGNAAAAAATGCTTTATGATG 152
4567 GGACAAACAC...AATAG.AATGCAAGTGAAGAAATGCTTTATGATG 4612
153 TGAATATTGTGATGCTATTGCTTTATTTGNAACCATTAAGCCGCAAT 202
4613...AAATTTGTGATGCTATTGCTTTATTTGNAACCATTAAGCTGCAAT 4659
203 AAACAAGTTAAACAACAACAAATTCATTATTTATGTTTCAGGTTTCAGGG 252
4660 AAACAAGTTAAACAACAACAAATTCATTATTTATGTTTCAGGTTTCAGGG 4709
253 GGAGGTGTGGGAGG 266

M'79 (12) (211. seq) vs
M'79 BAC hrgN rev. BPNote some of pol A doesn't match perfectly.
Where did Dominic get the prot from?

(12)
Differ from
SV40 pol A
and
(16) seq
(L15)

Worse
214
seq

214
seq

1:50 pm
2:10 pm
0.8% HX4
NAX

M'79 BACHwGN.BP

Green cells Jan 2002.
but pure killed all

79

Can. Dec. 9th

M'73 (cont.)

Transf. media

BACHwGN BMHC PURD

Dye
W
AGMM

1/28

M'79 BH.5d

80 pl ✓

M'73 lig.

4 pl ✓

3:42 pm

4:32 pm

42°

90 sec ✓

Plated 1XNZY 1XAMP

Rest, 20, 5, 20, 5, 1
16, 16 9 am

Back to 37° 5 out at 11:30
37° 4:42 pm

Minis

24 picked (12 from 5/16 & 20/16) including

24 w/6 smaller from 5. 1XNZY 1XAMP 37° 2:25 pm Mon. - 9 am Tues

ACR
W
Dye

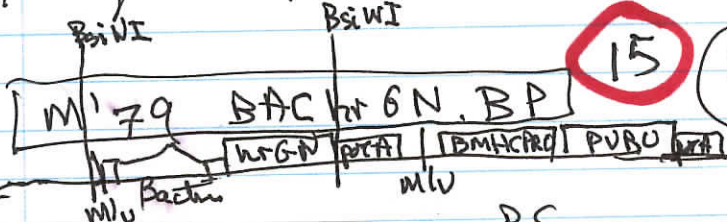
Take ✓ 50 pl 10XRI salt

✓ 445 pl 2dW

✓ 5 pl (50 u) BsiWI hot-18 (10 u/pl)

20 pl aliquots + 1 pl minis M'79 ① - ②④

37° 11:31 am



15

Red

M'79 Red

1:30 pm

give BsiWI
4 kb + 19 kb

Error left M'79 (12) → (15)
K17 Red Mlu/100
at room temp. overnight

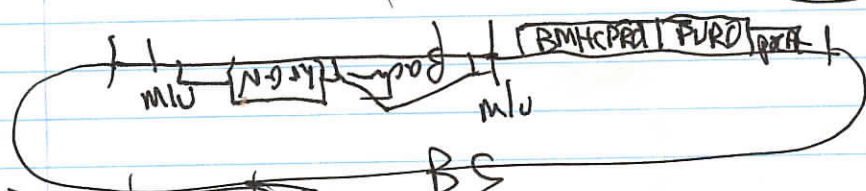
No sequence
214, 215

M'79 BACHwGNw. BP

12

Blue

M'79 Blue



Give BsiWI
9 kb + 14 kb

214 48	K17 Red Mlu/100 5 pl	M'79 12 2 pl	Water ✓ 13 pl	213.89	K17 Red Mlu ✓ 5 pl	M'79 15 2 pl	Water ✓ 13 pl
-----------	----------------------------	--------------------	---------------------	--------	--------------------------	--------------------	---------------------

- with insert to give more Clontech sequence.
- (i) Clontech pCFPm1 to Afl II to Sac II. Excise Nhe I into Cypm1
- (ii) (ii a) AMHC Sac I $\rightarrow$ Nhe I Bam HI to Xho Sac I Xho
Excise Nhe I AMHC Xho Sac I
- (iii) Assemble
-
- (iv) Excise with Hind $\rightarrow$ Xho for Cypm 3 assembly
- (v) To use with pSKBI ^{need} Mlu and BspEI

AMHC
Nhe-Hind
Sac
Bam

Sun. Dec. 9th

for HAT^R or 6TG^R

AMHC Neo

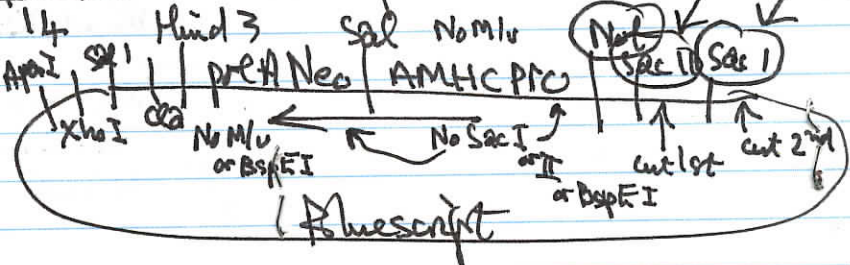
AMHC Mito Cyan

w'd be useful for selection & later mixing cardiomyocyte expts. with BMHC Puro / BMHC hGN

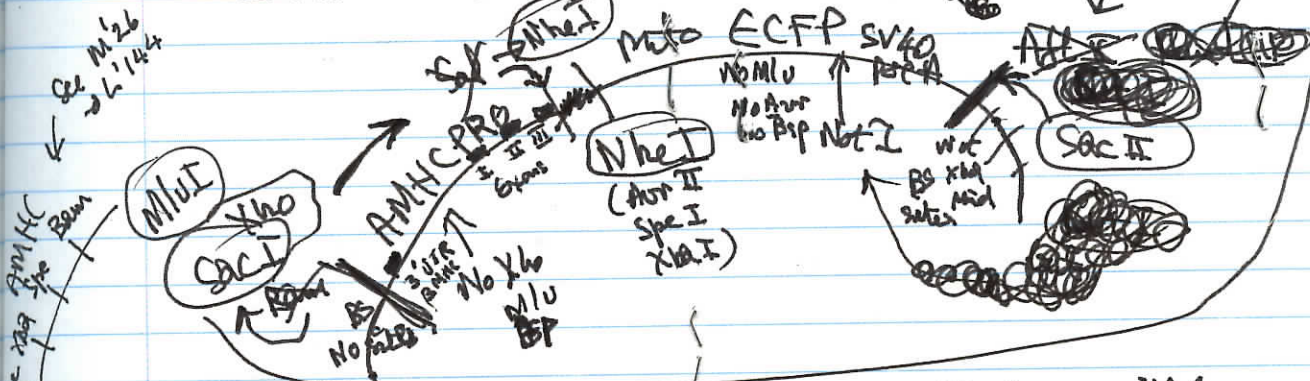
Can put this into usual cta construct or into exa 3 vector.

AMHC Neo is currently available

as M'14



AMHC Mito Cyan can be made from Clontech vector via AgeI to



Can still excise with Xho & Hind so will be a back up for m'23 & modifications as m'67.

Sat. Dec. 15th

M'33

#7 is old (?) Single site Age I Bhr GN. AC (cont'd)

- too much sample or misload - c'd be OK.

No

Yes

Age
+ Bam
minis
M'77

PCR

Hypothetical that the Yes site cuts best on Supercut, I

will start with Age I & then add Bam HI for

site diagnosis.

Take

✓ 40 μ l 10 $\times$ RI salts✓ + 310 μ l ddw✓ + 25 μ l (50 v) Age I lot 34 (20 μ /v)375 μ l (15 $\times$ 25)✓ 15 μ l aliquots + 1 μ l M'77 minis ① - ②④37 $^{\circ}$ 1:30 pmEach plus 4 μ l Bam HI mix: -✓ 12 μ l 10 $\times$ RI salts✓ 105 μ l ddw✓ 3 μ l (60 v) Bam HI lot 82 (20 v/ μ l)120 μ l (30 $\times$ 4)37 $^{\circ}$ 1:40 pm to 2:20 pm

Can sequence with M'29 Red Cyan
primer to confirm. It will not
sequence if the Age I site is missing.

(25010) +

Bam	Age	Both	site in Bam	site in Cyan
1680	3.9	3.9	3.9	3.9
4540	3.5	3.5	3.5	4.6
8078	1.02	1.02	1.02	606
9100	5.5	5.5	5.5	5.5
9168	6.5	6.5	6.5	1.00
14610	1.00	1.00	1.00	1.00
15408	10.1	10.1	10.1	10.1
(25010)				

Enter

#4 is

too short

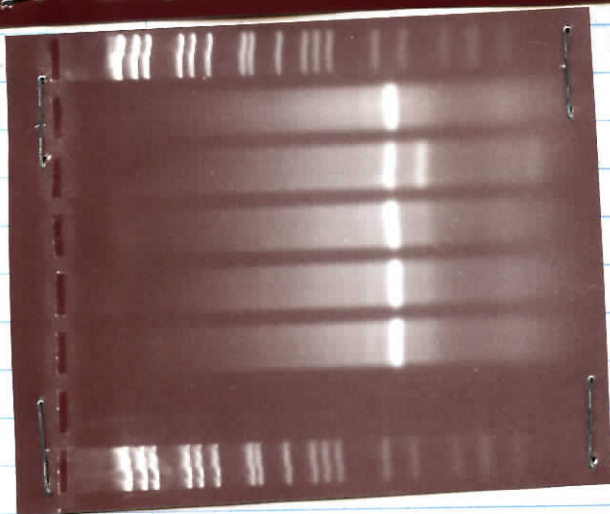
(see M'120)

4.3 kb Bam,

not 4.6 kb



①
Conclusion: Adequate
 product. Check
 digest. conditions
 for Age I

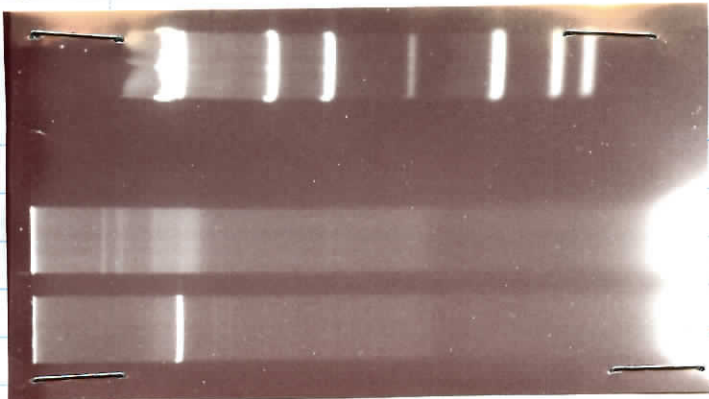


② Conclusion: Amazing! Only RT
 works. Product (incomplete) is
 OK. Proceed but add more enzyme
 after 2 hrs. & keep for 4 hr. total

AgeI
 |
 AAAAACC GGT gatccgctagcatgtccgtcctgacgccgtgctgctgctggggttgaca
 1 -----+-----+-----+-----+-----+ 60
 TTTTGGCCActaggcgatcgtacaggcaggactgcggcgacgacgacgccccgaactgt

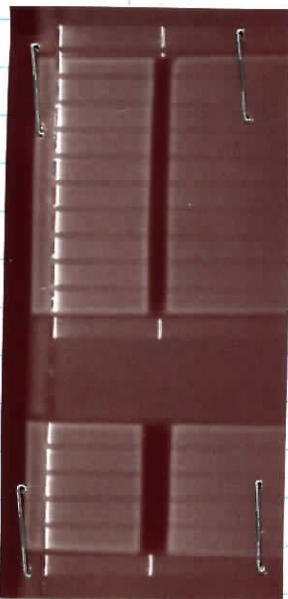
AgeI
 |
 ggctcggccggcggtctccagtgccgcgcgccaagatccattcgttgggggatccaccg
 61 -----+-----+-----+-----+-----+ 120
 ccgagccgggcccgcgagggtcacggcgcggttctaggttaagcaacccctagggtggc

gtcgcacccatggcctcctccgagaaacgtc
 121 -----+-----+ 150
 cagcgggtgtaccggaggaggctcttgcag

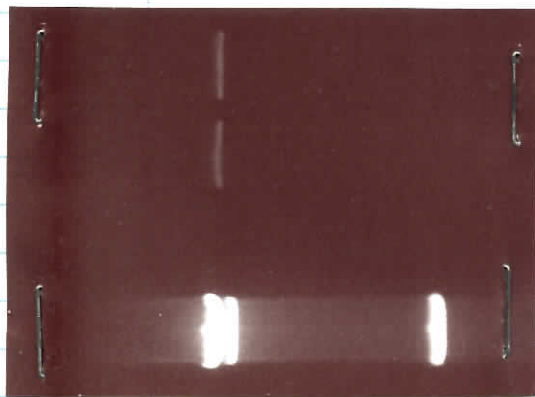


①

Conclusion: Sample rate cut
is fine.



② Good cuts.

③ Timing good, as expected,
but usable.

③ 7:15P
0.89 HX4
W/om

Bye
W
10+6 M'87 (4) Age/c
10+6 M'87 (4) Age
W
AG M M
W
Bye

On 1:20P
0.89 HX4
W/om

Bye
W
AG M M
W
Bye

②

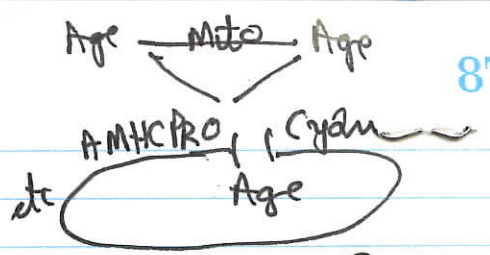
12.5

6

1:20pm
 12:20pm
 0.85g HX4
 1.3g max

Sat. Dec. 16th

Single site (contd.)



Agm W
 Pre M'87
 Post Age 4
 W
 M'87 Age 4

Take 20 μ l 10x RI salts
 140 μ l ddw
 20 μ l M'77 #4 mini
 20 μ l (40 μ l) Age I hot

320 11:25 am to 1:10 pm

6+10
 M'87 Pre
 6+10
 M'87 Post

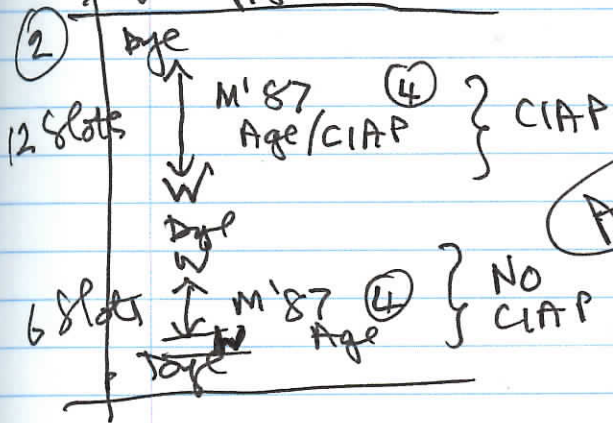
Note - if the PCR product cannot be cut with Age I can change to XmaI, BsaVI, BspEI, AatI - need to check with Kozak before choosing. No isoschizmer available from New England.

120 μ l M'87 Age 4
 30 μ l 1M Tris pH 8
 2 μ l lab CIAP

M'87 Age/CIAP 4

50° 5:14 pm to 5:46 pm

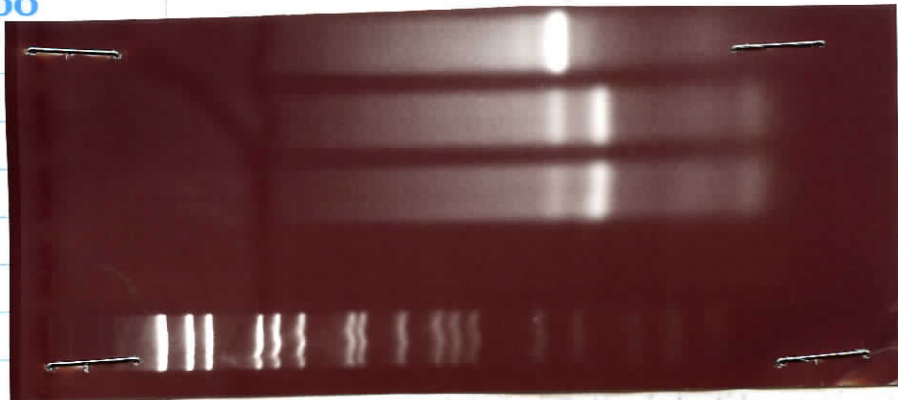
6:05pm
 12:05pm
 0.85g 1.5v/cm
 Agarose



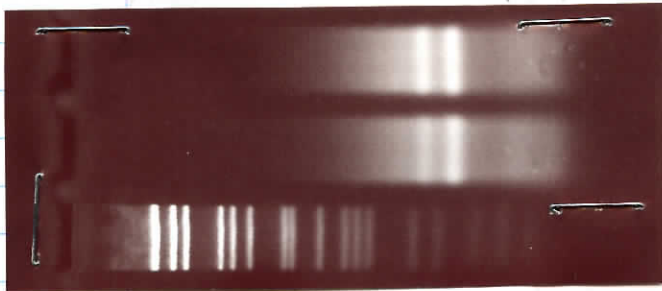
Agarose

Excized on 0.85g & 0.46g pieces + 1/10 8M NH₄Ac.
 Freeze thaw. Mash.
 Phenol extract at ~85°
 Recovered 800 μ l - 400 μ l
 Back up into 80 μ l as M'87 Age/CIAP
 40 μ l M'87 Age 4

Blue
 Red



① Conclusion: The 1x4hr looks better overall, since uncut well not interfere greatly even if $\frac{1}{2}$ cut.



② All is well, the 37bp piece may have melted.

Can check these by sequencing with M'61 Age Mito Blue primer ~~but not~~ with M'61 Red Red primer, since that was for ~~Red~~ Ad. Only correct orientation will sequence.

Picked to 1x Amp NZYminis ① — ⑩ Blue M'89 CIAP
⑪ — ⑫ Red M'89 NoCIAP 37° 1:05pm Thurs. to 9:15am

4 hr
all
only

2:55
off 3:35
210 sec 1000 rpm
H x 1

Tues. Dec. 18th Age Mito Age
B hr G.N.A Age Age (cyan)
SKBI

Age w ①
ACR mm
w
PRE
4hr
4hr 2x
w
Dye

Assembly

PCR digest

M'89 Take 40 pl M'85 PCR product
16 pl 10x RI salts
100 pl H₂O
4 pl (80) Age I hr 34 (20/pl)

16 pl
PRE M'89

R.T. 10:23^{am} to 12:15^{pm}
2 pl map to 2:40^{pm}

Age w ②
4hr
4hr 2x
ACR
Age

Post heat

Ligation

M'87 ④
M'89 4hr (1x) 3 pl
(heat inact!)
10x p95 lig. buffer 3 pl
M'89 T4 ligase 3 pl

CIAP
M'89 ligⁿ
Blue label
(CIAP)

NO CIAP
M'89 ligⁿ
Red label
(NO CIAP)

Get & ice
Heat inactivate
prior to use.
58° 280 min
got a bit high.
Check again by
ep prior
to ligation.

20 pl Age/CIAP
20 pl Age
3 pl
3 pl
3 pl To cold at 5:40^{pm}

Can distinguish
with BamHI

No mito
(6.4 kb)

Mito & Reverse Mito
(5.6 x 1.0) (5.5 x 1.1)
Wed. 5:15^{pm} perhaps if minis are good.

Transformations

Blue Age/CIAP ligⁿ 80 pl
Red Age ligⁿ 80 pl
Plated 1x AMP NZY
Rest 20, 5, 20, 16, 5
M'89 Blue
M'89 Red

1:55am Fri

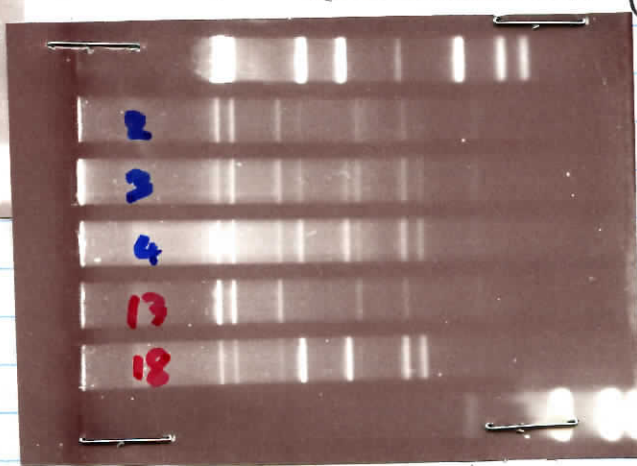
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

(1)
Conclusion: The main product ~~is~~
~~is~~ agrees with my map. ~~Many~~
~~Red~~ ~~Red~~ ~~Red~~
 (2) (3) & (4) have something near
 5.5 (13)
 Re test with better spin
 & more DDA

(1) - (10) Blue discarded.
 Continued with (18). Pooled
 others like (18) in place
 Single Agent, of M'77 #4

(2) Conclusion: 5.7 of (18)
 (18) is best. (2) & (13) are
 just possible. Try other
 conditions for the Dam digest?
 (2) Re-interpretation (18) has 6.6 so is wrong
 (13) has 6.6 but lacks 10.1 so is not good but
 could be digest. Try better digests (2)

Pool (1) (12)
 (14) (15) (16) (17) (19) (20) (21) (23) (24)
 as M'89
 Red Pool



On 1:25 pm
 9.80 Hk4
 Max + 3rd 10th
 RNAM

Fri. Dec 21st

Blr G.N. Amito/Cyan
 Age Age

M'81 Age insert (contd.)

(24) Bam
 M'81
 num

M'81
 Minus proceed to 50 pl.

Take ✓ 50 pl 10x RI salts

✓ 448 pl ddw

✓ 2.5 pl (50^u) Bam HI hot 82 (20^u/pl)

W 20 pl aliquots ~ 2 pl of minus M'81

37° 11:26 to 12:45 pm

On 1:25 pm
 9.80 Hk4
 Max + 3rd 10th
 RNAM

On 1:25 pm
 9.80 Hk4
 Max + 3rd 10th
 RNAM

On 1:25 pm
 9.80 Hk4
 Max + 3rd 10th
 RNAM

On 1:25 pm
 9.80 Hk4
 Max + 3rd 10th
 RNAM

On 1:25 pm
 9.80 Hk4
 Max + 3rd 10th
 RNAM

On 1:25 pm
 9.80 Hk4
 Max + 3rd 10th
 RNAM

On 1:25 pm
 9.80 Hk4
 Max + 3rd 10th
 RNAM

On 1:25 pm
 9.80 Hk4
 Max + 3rd 10th
 RNAM

On 1:25 pm
 9.80 Hk4
 Max + 3rd 10th
 RNAM

On 1:25 pm
 9.80 Hk4
 Max + 3rd 10th
 RNAM

On 1:25 pm
 9.80 Hk4
 Max + 3rd 10th
 RNAM

On 1:25 pm
 9.80 Hk4
 Max + 3rd 10th
 RNAM

On 1:25 pm
 9.80 Hk4
 Max + 3rd 10th
 RNAM

On 1:25 pm
 9.80 Hk4
 Max + 3rd 10th
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On 1:25 pm
 9.80 Hk4
 Max + 3rd 10th
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On 1:25 pm
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 Max + 3rd 10th
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On 1:25 pm
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 Max + 3rd 10th
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On 1:25 pm
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 Max + 3rd 10th
 RNAM

On 1:25 pm
 9.80 Hk4
 Max + 3rd 10th
 RNAM

On 1:25 pm
 9.80 Hk4
 Max + 3rd 10th
 RNAM

On 1:25 pm
 9.80 Hk4
 Max + 3rd 10th
 RNAM

On 1:25 pm
 9.80 Hk4
 Max + 3rd 10th
 RNAM

On 1:25 pm
 9.80 Hk4
 Max + 3rd 10th
 RNAM

Dec 1st assembly

No insert

Correct

Reverse

Main Obs.

682 10.1

682 10.1

682 10.1

10.1 ✓

4540 3.9

4540 3.9

4540 3.9

~4 ✓

9102 4.6

9102 4.6

9102 4.6

4.4 ✓

9165 63 bp

9165 63 bp

9165 63 bp

6.6 ✓

9165 6.6

9165 5.55

9165 5.45

6.6 ✓

14716 1.00

14716 1.00

14716 1.1

6.6 ✓

15720 (10.1)

15720 (10.1)

15720 (10.1)

6.6 ✓

(25122)

(25122)

(25122)

6.6 ✓

(25.1)

(25.1)

(25.1)

6.6 ✓

length

96 pl

Take ✓ 12 pl 10x RI salts

✓ 82.5 pl ddw

1.5 pl (30^u) Bam HI hot 82 (20^u/pl)

16 pl + 4 pl of minus M'81 (2) (3) (4) (13) & (18) (2 pl)

37° 2:10 to 2:05 pm

Sat.

Sequence with M'29 Red Cyan primer to check (2) (13) & (18)

Hold further work until this is done.

Should also consider (2)

See M'81

Oligo # 31766 5' Oligo Name: M'92 Hind BspEI Hind 3'

Date made: A G C T T T T C C G A A I

LENGTH: 14

% G+C: 43%

Oligo # 31767 5' Oligo Name: M'92 ~~Not~~ SacII ~~Not~~ 3'

Date made: T T A A A A C C G C G G T T

LENGTH: 14

% G+C: 43%

Oligo # 31768 5' Oligo Name: M'92 ~~Not~~ ~~Not~~ ~~Not~~ 3'

Date made: G A T C T C T C G A G C C G C

LENGTH: 36

% G+C: 61%

Oligo # 31769 5' Oligo Name: M'92 Blue ~~Not~~ Clontech ~~Not~~ 3'

Date made: T C G A A C C G T C A G A T C

LENGTH: 22

% G+C: 59%

Oligo # 31770 5' Oligo Name: M'92 Red ~~Not~~ Clontech ~~Not~~ 3'

Date made: T C G A G C T A G C G G A T C

LENGTH: 22

% G+C: 59%

✓ M'92 for M'23 This is for exaction of pieces for pSKB for Exon3

4441 CGAATCTCTCTGATCTTAAGCCCCACTAGGTTTATATTCTATATTTGATATCGAATTC 4500

GCTTAGAAGGACTAGGAATTCCGGGGTGATCAAATATAAGATATAAATATAGCTTAAG

MLuI NotI

ACGCGTGCTATGCGGCGCGGAGAACTCTCGTAAAACTCTCATGGCAGTAGTTATTAT

4501 TGCGCAGCATACCGCGCGCGCTCTAAGAGCATTGTGAGAAGTACCGTCATCAATAATA 4560

TCTCTCTCTCTCTTTCTTTCTTTCTTGAGCAGGATTTCTCTGTTGCCAGGCTG

4561 AGAGAGAGAGAGAGAAAAAGAAAGAACTCTGTCTATAAAGAGACAACGGTCCGAC 4620

GAGTGCAGTGGCAGACTCTGGCTCACTGCAGCTGGACCTCTGGGCTCAAGCCATCT

4621 CTCACGTCAACGCTGACAGCAGTGACGTCCGACCTGGAGACCCGAGTTCGGTAGGA 4680

PSKB1.AVR II

CCATTTGCTGCTGACTGCTCTAAGTCAATATATATATACACACAGGCATACCTA

5101 GGTAAACGACTGACATGACAGGATTACGTATATATATATGTGTGCGGTATGGAT 5160

TACTCCTCTAACTCAAAATGATCCTGTTATGTTATGTTAATATGCTCATTTTATTT

5161 ATGAGGAGATTATGAGTTTACTAGCAATATATACAAATATACGAGTAAAAATAA 5220

TAAATTTTATTTATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTT

5221 ATTTAAAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAA 5280

SmaI XmaI

AGGCTGAAGTGAGTGGTGGCATCTCAGCTCAGTGGGACCTCCGCTCCGGGTTCAAGT

5281 TCCGACTTCAGCTCAACAGCTAGAGTCAGTCAAGCTGGAGCGGAGGGCCCAAGTTCA 5340

BspEI AvrII BssHII

GATTCTCTGCTCAGCTCCGGAACCTAGGTTCCGGGTAAGCCGGGCGGGGGGGGGGG

5341 CTAAGAGACGAGTCAGGAGCTGGATCCAGGCCGATCCGGCGCGCCGCCCGCCGGCC

PCR1 AMHCPRO-NEO

modified

6330 → Hind BspEI Hind

SpeI

Amu HI

AMHCPRO-NEO

BS/Aur

P-Mito

eII

ab

ARE II Sac II Age II

.AN

← This is for use with HAT

5' hand AMHCPRO.mito CYAN AMHCPRO-NEO 3' hand

MLuI NotI BspEI

BS

Use

5'107

PSKB1.AVR II

for cloning

indicates that the sequence has been assembled in OCG.

Sat. Dec. 22nd - Wed. Dec. 26th

Fragment

De look at M13/Amto C. AN (see M'87 M'26 L'144)

Goals: -

(A) M'92.2 1084 bp

Change In progress

(i) start with pECFPmto from Clontech.

Change Afl II to Sac II. (M'92.2)

Excise (Nhe I) ECFPmto pEA (Sac II)

(ii) start with original AMHC clone

and change Bam to

Sac I Mlu I Xho I Bam

(iii) then change Sal to Sac Clontech 5' sequence Nhe I Sal

JL6-23

(iv) Improvements Excise (Sac I) AMHC Clontech (Nhe I) (M'92.3)

Pre (vi)

(C) M'92.4 9.3 kb

(v) change Hind to Hind BspE1 with M'92 Hind BspE1

Start with M'14 pEA Neo AMHC PRO Sac II Sac I cut 1st with Sac II 2nd with Sac I

M'92.4 (after cutting)

(vii) Assemble

clg pEA Neo AMHC PRO Xho Hind

(Sac II) (Nhe I) AMHC PRO (Sac I) (B) (Sac I) (A) (Nhe)

M'14 Modified

Check ability to excise Xho - Hind for

Need to change Hind in M'14 to Hind BspE1 Hind for pSK B1 insertion via Mlu BspE1

First step

AMHC linker

GATC CTCGAG ACGGTGAGCTC ACGGTCTCGAG
M'92.2 1084 bp
Ex Bam Xho I Mlu I Sac I Mlu I Xho I Ex Bam

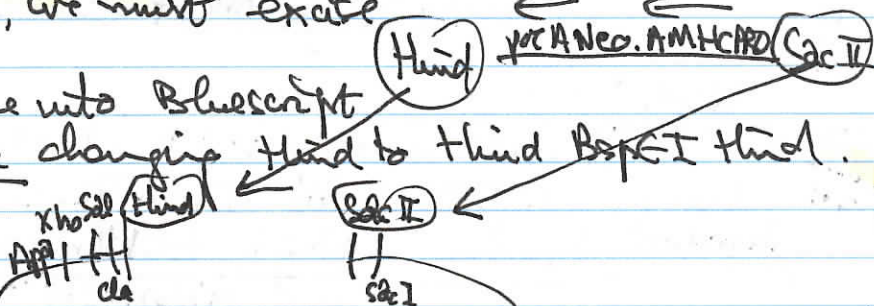
Clontech linker TCGA ACCGTC AGA TCCGCTAGC

ex sal Clontech Nhe I ex sal

Step (v)

Comments: A problem may be present with the Sac I site in m14 AMHC.NEO - it is probably not next to Sac II. If that is so, we must excise

→ redone into Bluescript before changing Hind to Hind Bsp I Hind.



Made but not used

BS

M 94 AMHC.NEO

(in regular Bluescript)

JC6-12

✓ m14 checks for Sac I + Hind III
JC-9 → is ~ with Sac II + Hind III
JC-12

This was not necessary; M14 was already in BS regular.

Added Note

Feb 3rd

If Mito Cyan ~~is~~ Cyan, then
→ is not as good as
(Sac II) pCA ECFP Mito (Whe I)
will need to be replaced with

(Sac II) pCA ECFP ~~Whe I~~
→ Whe I

Thurs. Dec. 27

(Summary from M'92 & 93)

Steps to [M'92 for M'23]

- (i) Start with pECFP-Mito from Clontech
 Change ~~Afl~~II to ~~Afl~~II Sac II ~~Afl~~II
 with M'92 ~~Afl~~II Sac II ~~Afl~~II linker.
 Excise 1 kb (Nhe I) mito ECFP pA (Sac II) (M'92.2)

- (ii) Start with M'13 α MHC, and change Bam to
 Xho Mlu Sac I Nlu Xho with M'92 linker

- (iii) Then change Sal I to Sal Clontech 5' sequence Nhe I

- (iv) Excise (Sac I) AMHC PRO Clontech (Nhe I) 5.5 kb (M'92.3)

- (v) Start with M'14 AMHC, NEO and change Hind III to
 Hind BspEI Hind with M'92 Hind BspEI Hind to
 give M'92 AMHC, NEO modified

- (vi) Cut ~~with~~ M'92 AMHC, NEO modified
 first with Sac II and then with Sac I
 to give (M'92.4)

- (vii) Assemble M'92 from M'23 from 3 pieces: (M'92.2, M'92.3, M'92.4)



Can excise the whole region with Xho and Hind for Exon3 vector
 or with Mlu and BspEI for pSKB1.ANR II vector

96

Class	M'96 Am Xma I Am				
Date made	TTA	A A A	CCG	GGG	IT
Length					
Rec'd					

14
43%

This gets rid of "junk"

M'97. BhrGN.AmitoC

Fri. Jan 4

See also M'91 PCR attempt (Snow day ~ 9" on return from UK. over New Year)

97

M'94. BMHC hron. AMHC mito Cyto

M'21. BhrGN.AC

(i) Digest with XmaI

M'23. BhrGN.AC

unconnected

XmaI 11060

XmaI 16440

Xma

Xma

(ii) M'92 for M'23

Change SpeI 6570

This allows removal of "junk"

(iii) Excise

XmaI 1920

HC PRO. mito C pol A XmaI 6570

(iv) Clone into CIA-Ped M'33 BhrGN pot A AM

11060 XmaI

16440 XmaI

ACTAGAAA CCC GGG TTCTAGT
111 999 333 000 500

#	31787	Oligo Name:	M'97	SpeI	XmaI	SpeI
made			CTA	GAA	ACC	CGG
16			T			GTT
50%						

This will give me the animal equivalent to M'21 but with mito Cyto in place of Cyto cytoplasmic

Sat. Jan 5th

Can also make more directly without waiting for M'92 for M'23

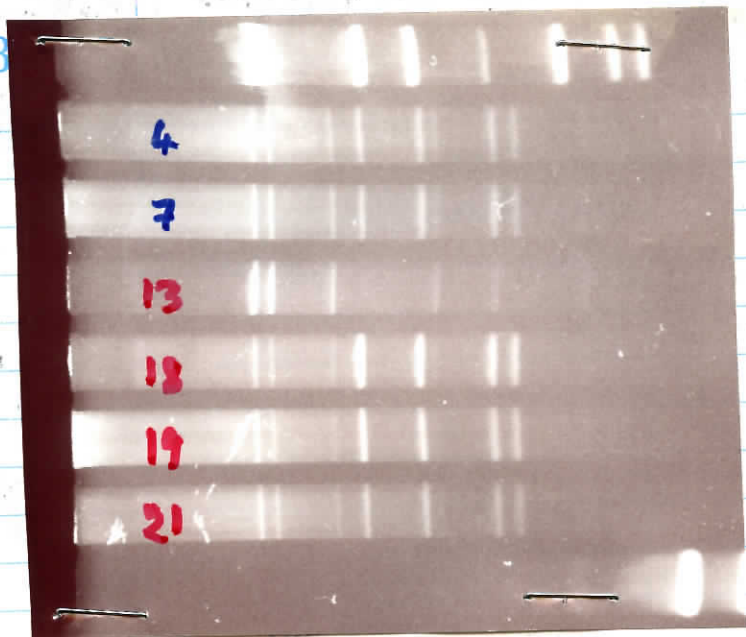
(v) M'13 aMHC SalI to SalI clonch SalI

(vi) Excise XmaI HC PRO clonch XmaI

(vii) Change AflII in pECFP-Mito to AflII XmaI

(viii) Excise XmaI

(ix) Clone into Xma BlueScript (vi)+(viii) & go to (iii)



① Conclusion: Not much additional information. Need to see if Age I is in correct place. It should cut the 6.5 kb band (Bam + I) into 5.5 + 1 kb.

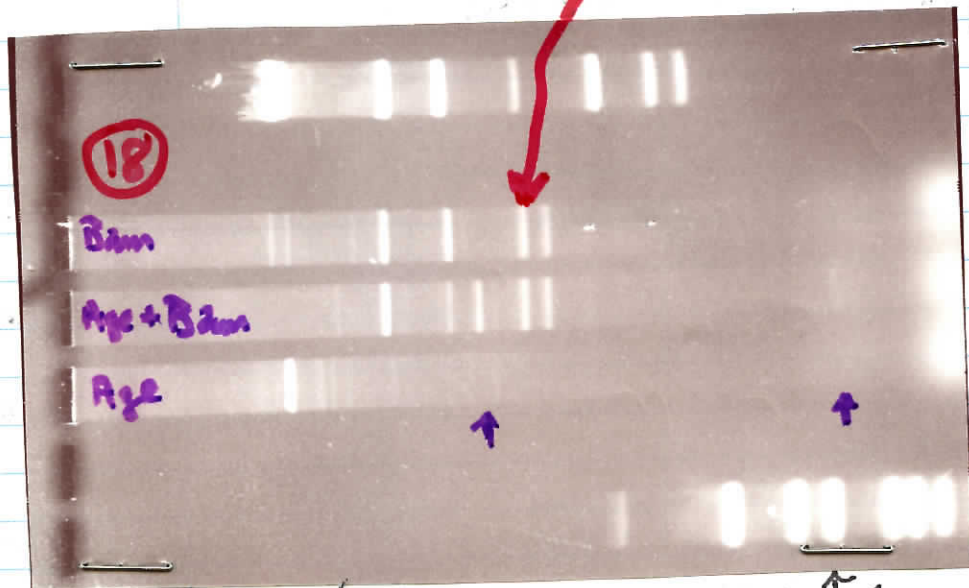
The wrong Age I site will cut the 4.6 kb band into 3.5 + 1 kb.

If both are present, we will see 2 x 1 kb, 3.5 + 5.5

? Error. See M'120.

The removed Age I site may have deleted ~250 bp.

This is 4.3 but sh'd be 4.6



② Conclusion:

(18) definitely has the correct Age I left → the other destroyed. Start from here again. Re-construct with M'89 ligation

on 4:30
Bam
w
PCR
(4)
(7)
(13)
(18)
(19)
(21)
Age
w
B
37 2

2:45 pm
M'89

216

On 4:35 AM 5:55 PM 0.8% Hx4 3/10 RNAse MAX

- Age ①
- W
- PCR
- ④
- ⑦
- ⑬
- ⑮
- ⑰
- ⑲
- ⑳
- ㉑
- AG
- W
- Age

Sat. Jan 5th

Re-look at M'91 candidates

Take 160
 ✓ 20 μ l 10x NEN buffer Bam HI
 ✓ 20 μ l 10x BSA
 ✓ 118 μ l ddw
 ✓ 2.5 μ l (50u) Bam HI lot 82 (20u/ μ l)
 ✓ 16 μ l + 4 μ l of mini M'89
 + 2 μ l of mini

- ④
- ⑬
- ⑦
- ⑮
- ⑰
- ⑲
- ㉑

These mini
 has been
 in cold
 since Dec. 21
 No problem

37° 2:10pm - 4:20pm

2:45pm 4:00pm Hx4 3/10 RNAse MAX

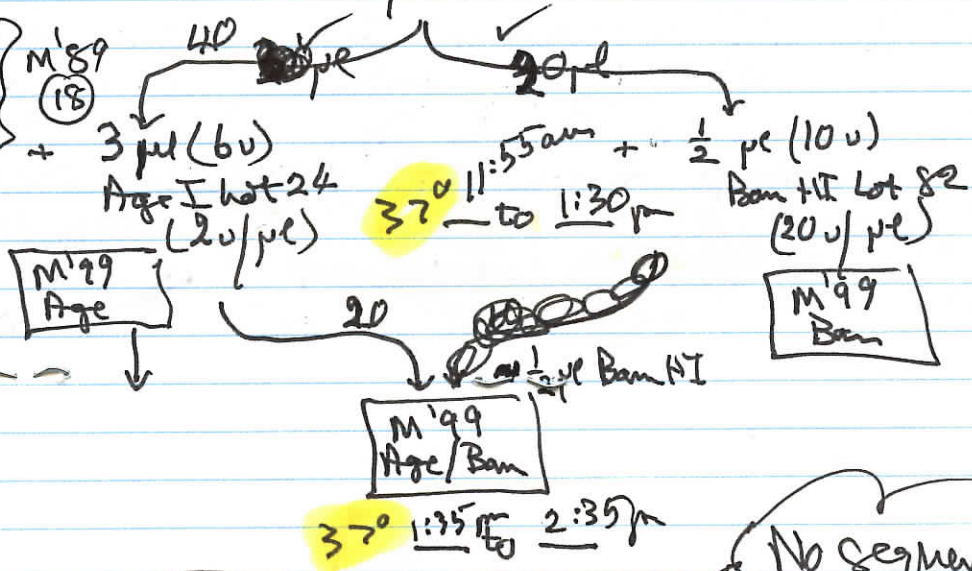
Sun. Jan 6th

Check on ⑮ Age I - site(s)

Age ②

Take ✓ 6 μ l 10x RI salts
 ✓ 48 μ l ddw
 ✓ 6 μ l M'89 mini ⑮

- AG
- W
- Bam
- A+B
- Age
- W
- PCR
- W
- Age



See M'85
 In future use
 room temp.
 for Age I

No sequence
 - poor primer choice

216 seq
 M'29
 Red/Green
 5 μ l 100

M'89
 5 μ l
 ⑬

Water
 10 μ l

215 seq
 M'68
 Age mito
 Blue/100
 5 μ l
 M'89
 5 μ l
 ⑬
 Water
 10 μ l

222 M'101 Red Cyan 2 M'89 (18) Water 14 pl Modest sequence

14521 aaagtcaggacttcacatagaagcctagccacaccagaaatgcacagacagacccctcc 14580
 ttccagtcctgaagtgtatcttcggatcggtgtggtctttactgtctgtctagggagga
 14581 atccccccataagagtttgagTCGAAGCACCGGTGCGCCACCATGTGAGCAAGGGCGAG
 tagagggggattctcaaaactcAGCTTCGTGGCGAGCGGTGTACCACTCGTTCCCGTC
 14640 GAGCTGTTACCGGGGTGGTGGCCATCCTGGTGGAGCTGGACGGCGACGTAACGGCCAC
 14641 CTCGACAAGTGGCCCCACCGGTAGGACAGCTCGACCTGCGGTGCGATTGCGCGGTG
 14700
 BclI M'101 Red Cyan 2 M'29 Red Cyan
 14701 AAGTTCAGCGTGTCCGGCGAGGGCGAGTGGCCACCTACGGCAAGCTGACCTGAAG
 TTCAGTTCGACAGGCCCTCCCGCTCCGCTACGGTGGGATGCGGTTTCGACTGGGACTTC 14760

Blue/100 2 pl 5 pl Water 14 pl Good sequence

Submitted Feb

224 M'101 BMHC M'89 (18) Water 14 pl No sequence

Blue/100 5 pl 1 pl

Results 223.seq shows clear AMHC sequences running into Cyan completely as expected with the Age I site off.

222.seq confirms this & extends into AMHC further.

AOK; proceed!

But see M'120 for trouble with site

222.seq check: 8159 from: 1 to: 818 /Reverse
 /afs/isis.unc.edu/home/n/o/nobuyo/oliver/M3.BhrGN.AC check: 610 from: 13901 to: 25011

ASSEMBLE September 6, 2001 18:55
 Gaps: 11 Quality: 1412 Ratio: 5.369 Score: 102 Width: 22 Limits: +/-23

290 CGCAGAGAACTCAGAC.....NAAATACATGGAGTCAATGGT 252
 14422 cacatagacctctgacagagagcaggacatttcatggagtc.ctggtg 14470
 251 GGAGAGGCCATAGACTANGTGTNANANAGGCAGTGAAGTGGTGTAN 202
 14471 ggaga.gccataggctcaggtgttaaaagaggcagggaagtgtgtgtag 14519
 201 NAAAGTCAGGACTTCACATANAAGCCTAGTCNNNGCCAGAAATNACAGA 152
 14520 gaagtcaggacttcacatagaagcctag..ccacacagagaatgacaga 14568
 151 CAGATCCCTCTATGTCCTCCCATAGAGTTTGAGTACAGAGCATCCGG 102
 14569 cagatccctctat.ctccccataagagtttgagT..CGAAGCA.CCGG 14614
 101 TCGCCAGCCTGCTGAGCAAGGGCGAGGAGTCTGTTCCATCCGGGTGAG 52
 14615 TCGCCA.CCATGGTGAAGAGGGCGAGGAG.CTGTC..ACCGGGT..G 14658
 51 GGTTCCTCCCTGGTANGCTGCTGG 26

223.seq check: 9464 from: 1 to: 902
 /afs/isis.unc.edu/home/n/o/nobuyo/oliver/M3.BhrGN.AC check: 610 to: 25011

ASSEMBLE September 6, 2001 18:55
 Gaps: 14 Quality: 5647 Ratio: 8.033 Score: 383 Width: 16 Lir

6 TAAGAACTGAGTCGAAGACCGGTGCGCACCTGGTGAAGGGGCGAG 55
 14591 taagagtttgagTCGAAGCACCGGTGCGCACCTGTGAGCAAGGGCGAG 14640
 56 GAGCTGTTACCGGGGTGGTGGCCATCCTGGTGGAGCTGGACGGCGAGT 105
 14641 GAGCTGTTACCGGGGTGGTGGCCATCCTGGTGGAGCTGGACGGCGAGT 14690
 106 AAACGGCCCAAGTTGAGCTGCTCCGTCGAGGGCGAGGGCGATGCCACC 155
 14691 AAACGGCCCAAGTTGAGCTGCTCCGTCGAGGGCGAGGGCGATGCCACC 14739
 156 TACGGCAAGCTGAGCTGAAGTTCATGCAACACGGCAAGCTGCCCT 205
 14740 TACGGCAAGCTGAGCTGAAGTTCATGCAACACGGCAAGCTGCCCT 14789
 206 GCCCTGGCCACCCTCGTGACCACTGACCTGGGGCGTGCACTGCTTCA 255
 14790 GCCCTGGCCACCCTCGTGACCACTGACCTGGGGCGTGCACTGCTTCA 14839

Mon. Feb. 4th

Primers for PCR & sequencing

Recheck of single site Age I on M'89 (18)

No sequence
221 seq

M'29 Red Cyan	M'89 (18)	Water
✓ 100 5 pl	✓ 2 pl	13 pl

still not a good choice - since Age I is so close

pECFP-mto M21-Brown AC clontech 781 14699

M'101 Red Cyan 2

CCG GAC ACG CTG AAC TTG T

44
16
Ln

Sequencing

This will be better than M'29

Red Cyan & can use this also for PCR of Age (Mto) Age (3' primer)

32147	CCG	GAC	ACG	CTG	AAC
19	TTG	T			
58%					

Improved 5' primer will use BspEI in place of Age I for M'61 Age Mto Blue

PCR

AAAA TCC GGA GATCCGCTAG CAT GTC CGT

BspEI

met clontech 606 pECFP-mto

32149	M'101 BMHC Blue	GAT	GCG	GCA	CTC	TGC
18						
79%						

32148	M'101 Bsp Mto Blue	AAA	ATC	CGG	AGA	TCC
29		GCT	AGC	ATA	TCC	GT
52%						

For BMHC in M21, try sequencing

GATGGG GCA CCTGCTGC

M'101 BMHC Blue

7998 M21. Brown AC

Sequencing

for AMHC in M21, try sequencing

TACAGACAGATCC CTCCTA TC

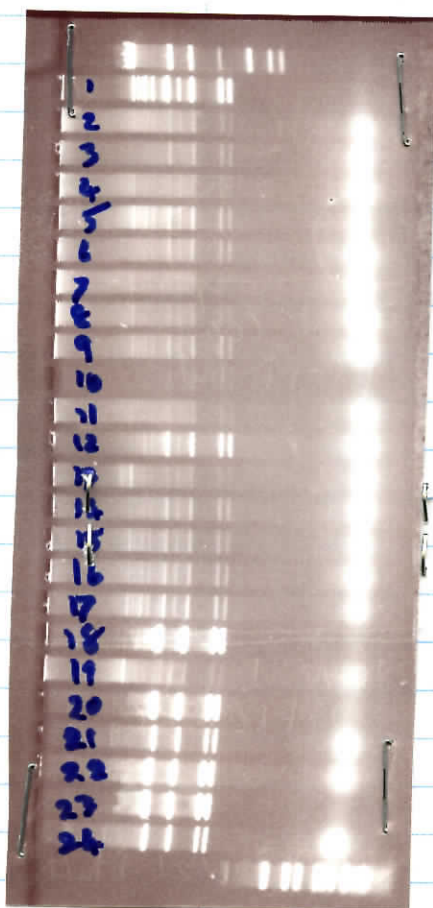
14583 M21. Brown AC

Comment: I cannot reconcile the sequence of the m'6 Red Red sequence (from IS Red ② with my use of IS Red ① for PCR). Wait! - before proceeding -

Second thought: the PCR product w'd not have digested & given a smaller product unless the 3' end was okay. Take a chance & proceed. Run a gel on Age / CIAP



① Conclusion: Cut is incomplete, but is as good as at 37°. Proceed & expect to do many runs.



② Conclusion: All are sticky - as expected. Amount looks to be related to growth - more DNA with less growth as judged by insoluble material.

Not very encouraging.

Try to purify again or retransform with m'89 (CIAP Blue) lig

Pool ① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⑨ ⑩ ⑪ ⑫ ⑬ ⑭ ⑮ ⑯ ⑰ ⑱ ⑲ ⑳ ㉑ ㉒ ㉓ ㉔ & re purify Age CIAP

ay 10:32
~~11:18 am~~
 0-89 Hx4
 man

Man Jan 7th

Age Age

103

M'91 Bhr GNA mto C(yaw)

Age ①

Re-attempt at M'91 without purification

W
 Age
 W
 M'103 (18) Age/CIAF
 W
 Dye

Take ✓ 6 μ l 10x RI salts

✓ 45 μ l ddw
 ✓ 6 μ l M'89 mini (18)
 ✓ 3 μ l (60) Age I lot 24 (20 μ l)

M'103 (18)
 Age

R.T. 1^{pm} to 4^{pm}

→ +15 μ l 1M Tris pH8 ✓

+2 μ l lab CIAF ✓

50° 4:10^{pm} to 4:45^{pm}

ϕ OH/HOQ, 2K CIAA

M'103 (18)
 Age/CIAF
 CIAA

M'99 215 seq
 Hold pending sequence

~~11:18 am~~
~~0-89 Hx4~~
~~man~~

M'103
 lig?

✓ 8 μ l M'103 (18) Age/CIAF/CIAA

✓ 10 μ l M'89 4hr (heat inact.)

← Maybe faulty

✓ +2 μ l 5M NH₄Ac

✓ +50 μ l EtOH Freezc. Cent. Dry. (no blind!)

Age ②

AC

Back up into

10 μ l p95 1x buffer ✓

① Bam lig. p95
 M'103 minus
 ① - ②4

15° +1 μ l M'77 T4 ligase ✓

Tues. 10:20^{am} to 3:20^{pm} RT 3:20^{pm} to 4:15^{pm}

②4

ACR

Transformation

37° 4:15^{pm} to 5:20^{pm}

W

Age

M'79 DH5 α

80 μ l ✓

32° 6^{pm} - 9:30^{am}

M'103 lig.

5 μ l ✓

1x NZY 1x AMP
 dry plates - pour spreads.

0° 5:22^{pm} to 5:42^{pm}

Rest, 20, 5, 20, 5
 many > 20 ⑩ ④

(Wed)

M'103 minus R.T. 9:45^{am}

90 sec. 42°

① - ②4 1x AMP NZY

32° 2^{pm} - 8^{am} cold

Enough for 24 more

Take 50 μ l 10x RI salts (Thurs)

20 μ l + 2 μ l M'103 minus ① - ②4 37° 2:45^{pm} to 3:45^{pm}

✓ 45 μ l ddw

5 μ l (1000) Bam HI lot 82 (200 μ l)

31809
 16
 50%

M'105 NheIBqEI NheI				
CTA	GCT	TTC	CGG	AAA
G				

31810
 16
 50%

M'105 NheI BsiWI NheI				
GGC	CAA	ACG	TAC	GTT
T				

Tues. Jan. 8th

Summary of meeting

with Julius A., Wayne C., Joe B., Dominic C., and Scip H.

Scip will work with Kimberly to get an animal from

BMHC LGN | BMHC PURO ←

Joe will try BATs (collagen ternia cultures) with

BMHC LGN | AMHC Cyan ←

Julius will work on culturing, pinching animals.

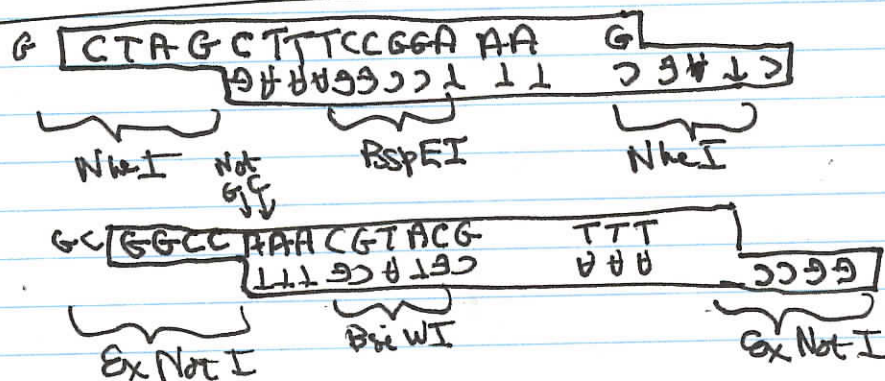
Joe will try to get dispersed cultures with

OS. will put a high priority on for no mito OK without mito

PSKB1 | AMHC Neo | AMHC mito Cyan but this first before mito

Scip can also test mito EGFP as for signal, toxicity etc.

in ^{Age I BspEI} Coletest. To do this will need to change NheI to ~~NheI~~ BspEI for Age I
and NotI (3' to Stop) to BsiWI (5' to Not)



Ex Spe I Not T Aat II

A [CTA G A A G A C G T C T T T] ●
 [L L L L L G G G G G H H H H H L L]

M'107 ~~Spe I~~ ~~Aat II~~ ~~Spe I~~
 C T A G A A G A C G T C T T
 16 T
 38%

M'107
~~Spe I~~ ~~Aat II~~ ~~Spe I~~

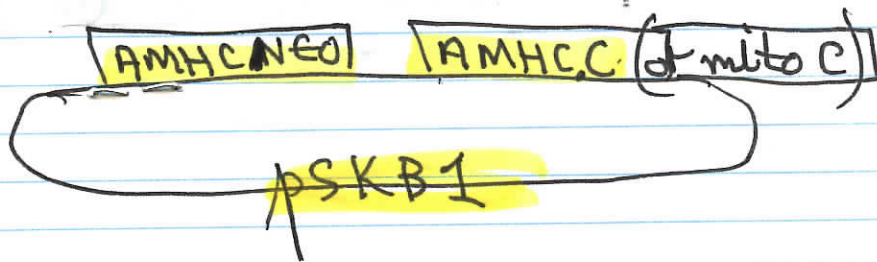
Not T Not I

From
 Kim

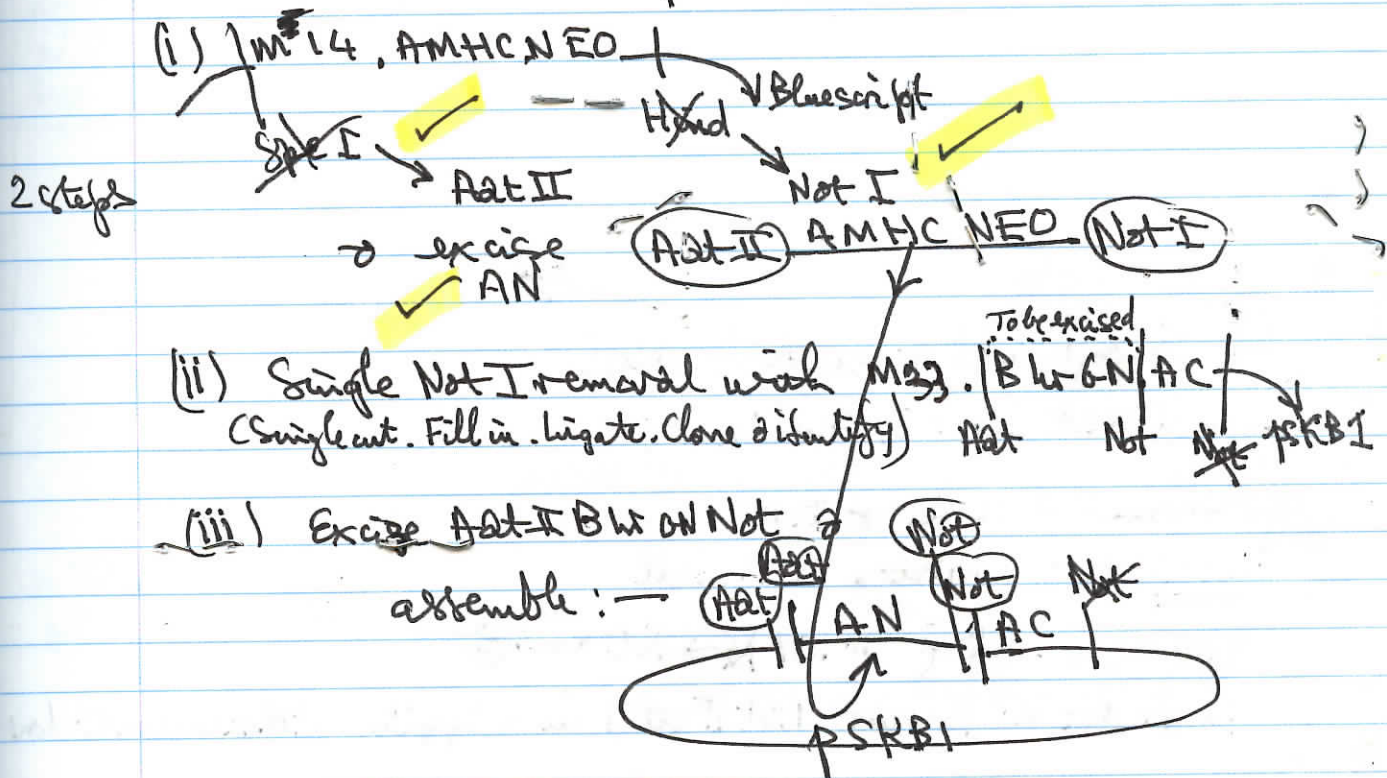
A [A G C T A G C G G C C G C T]
 [L L L L L G G G G G H H H H H L L]

M'106 ~~Not T~~ ~~Not I~~ ↑

Wed. Jan. 9th

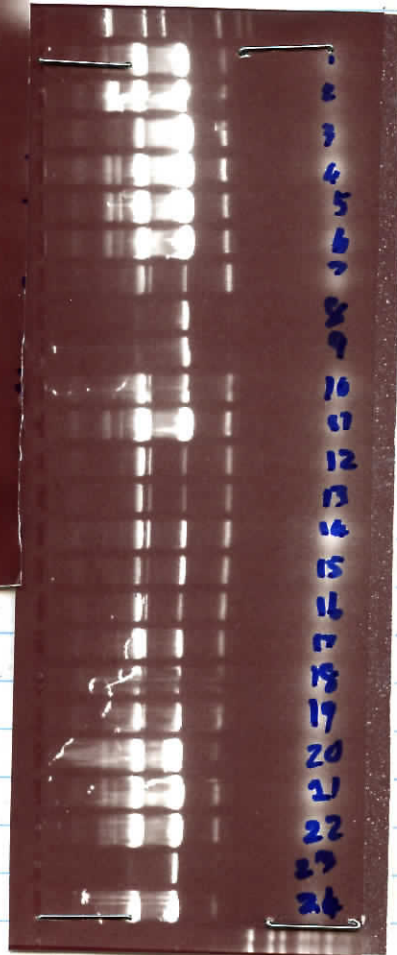
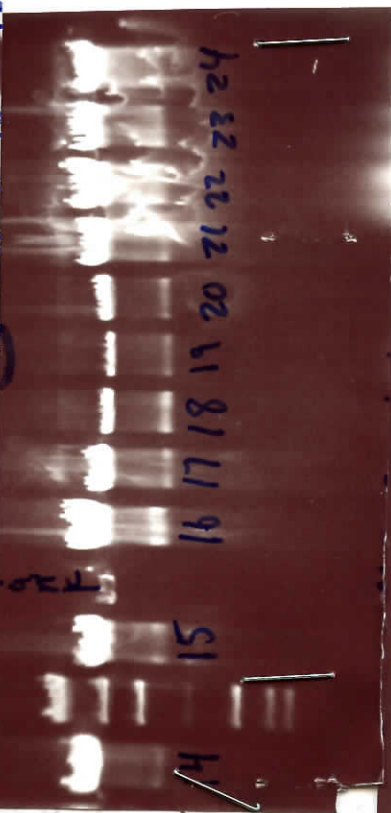
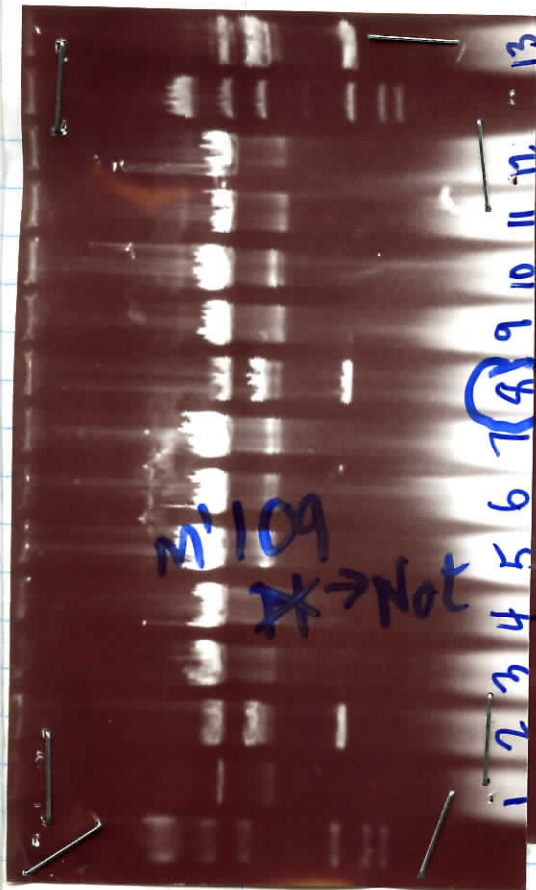


Can get to AN.AC : —
pSKB1



J.C. processed & out with
Not-I 01/11/02.

#'s (2), (8) & (13) are ok
as M'109 $\rightarrow$ Not-I



(2) is difficult to interpret. Most likely it that there is a mixture of monomer & dimer & that none are correct. T.H. will

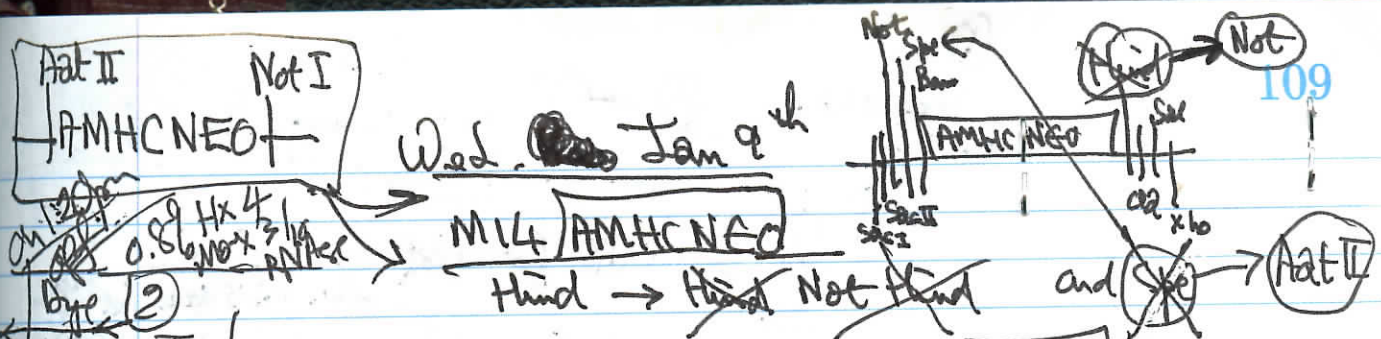
try again using M'109 $\rightarrow$ Not mini (8)

There are no known AatII sites in original AMHC promoter clone

Man Take 50 μ l 10x NEB buffer 4
✓ 445 μ l dH₂O
✓ 5 μ l (100v) AatII lot 48 (20v/ μ l)
37° 12 min - 1 min
M'109
1+20 Mini
SA (1) - (24)
(Spe Aat)

Sum M'109 Spe $\rightarrow$ Aat mini (1) - (24) packed v noan To 37° 4:25 pm
8:15 am
coll

If product is okay, excise AatII AN AMHC NEO pA Not-I



Wed. Jan 9th

M14 AMHC NEO

Hind → Hind Not Hind and Spe → Aat II

On 12/29/83
0.8 Hx 4
Not I
bpe (2)
W Aat II
Ag 10x buffer
M'109 SA
mini
24
AC
W
Dye

Take

- ✓ 6 µl 10x RI salts
- ✓ 52 µl ddw
- ✓ 2 µl M'14 AMHC. Neo #1 T.H. 4-83
- ✓ 1/2 µl (100) Hind III lot 52 (20u/µl)

37° 3:05pm to 3:50pm

Heat inact. 80-85° 10 min.

1bpe
M'109
PRE

- ✓ 10 µl M'109 Hind digest
- ✓ 1.6 µl p95 10x buffer
- ✓ 3 µl M'106 Hind Not Hind oligo
- ✓ 1.5 µl T4 ligase M'77 10pm 15° 4: to 4:55pm, 37° 4:55pm to 5:25pm

M'109
lig.

(Spe) Transformation 1x NZY Amp

M'79 DH52 80 µl R 20 5 20 16 5
(M'109 Spe lig) M'109 lig x 3 µl many (22) (4) 5:45 (4:05pm) 37° 6pm - 11am

M'109 minus (24) NZY + KAMP 37° 12:30pm Cold ~ 10am Fri

(3:35) 0° 5:25pm to 5:45pm (4:05pm) 37° 6pm - 11am
90sec M'109 Spe R, 20, 5, 20, 16, 5 4:15pm
many (12) (1) (Sat) Jan 12

Sat. Jan. 12th

- ✓ 6 µl 10x RI salts
 - ✓ 50 µl ddw
 - ✓ 4 µl M'109 Hind → Not mini #8
 - ✓ 1 µl (100) Spe I lot 28 (10u/µl)
- 37° 10am to 11am

16 µl
M'109
Atr Spe

M'109
Spe (8)

- ✓ 10 µl M'109 Spe (8) digest
- ✓ 1.6 µl p95 10x lig. buffer
- ✓ 3 µl M'107 Aat II Spe oligo
- ✓ 1.5 µl T4 ligase M'77 15° 10am to 11am, RT 11:40pm, 37° 3:10 to 3:30

M'109
Spe (8)

Spe
Transformation

① Conclusion: ① & ①9 are okay; ②2 may be an uncut dimer.

5 kb

Use ① at ①9 for ~~Not~~ → Bsi WI
with M105 Not Bsi Not

Then lost ~~the Bsi WI~~
~~into the CTA AAT Bsi~~
in K145 Age | Bsi CTA C1AA.

(True Bsp GI) into ECFP Bsi WI frag.
will go into Age - (Bsi) cta

← ①9

04:55 PM

Age
W
AG
①
24
ACR
W
Dye

(S)
Pick
X KAN
R

11:55 pm
 0.5 ml H₂O
 0.5 ml H₂O
 0.5 ml H₂O

Wed Jan 9th

Expression tests **mito ECFP**

NheI BspEI NheI
 into ECFP / pCA / APT
 KAN

Take 6 pl 10x RI buffer

52 pl Ld w

2 pl (100) M'III

1 pl (5) NheI

37° 3:45 to 4:45 pm

Inact! 80-85° 10 min

✓ 10 pl M'III M₁₀₅ digested

✓ 1.6 pl p95 10x buffer

✓ 3 pl M'105 NheI BspEI NheI

✓ 1.5 pl M'77 T4 ligase

Cold room 5 pm

Kanamycin

Transformation

M'79 8152

M'III ligase

0.3 pm

42°

80 pl

3 pl

3:25 pm

90 sec.

plated R, 20, 5, 20, 5

1x Kanamycin NZY 37° 3:37 pm

Cold 10 am (Fri)

Sat 10:40 am

Picked M'III minis ① - ② to

X KAN NZY RT until

Proceeded to M'III minis ① - ②

Take ✓ 50 pl 10x RI buffer

✓ 440 pl Ld w

✓ 10 pl (100) BspEI lot 3 (100 pl)

20 pl + 1 pl 37° 4:55 pm (Cold)

Fun

Revised old 1996

24 correct.

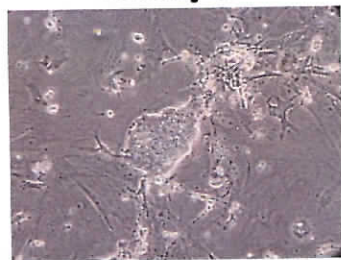
x20

MitoCcta, Day 6 after electroporation

Colony 1

Colony 2

Bright field



Cyan filter



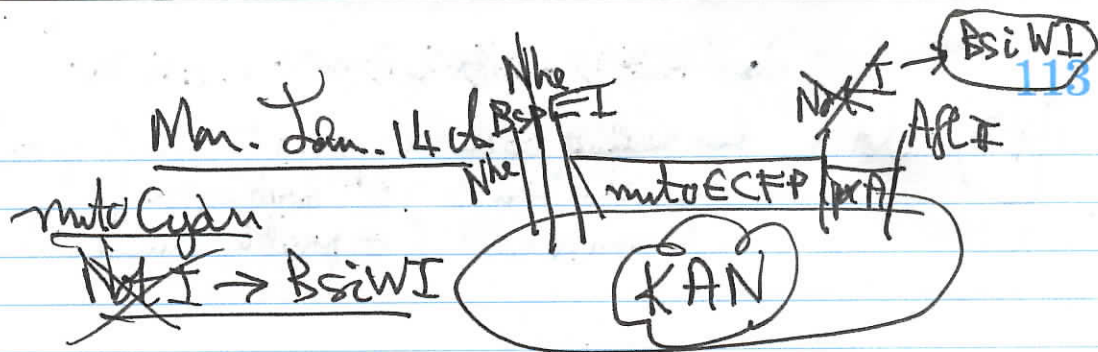
Yellow filter



Red filter



x20, exposure time = 1/2



Take 6 pc 10x RT salts

✓ 52 pc ddw

✓ 3 pc M'113 (19) mini

✓ 1 pc (10 v) NotI hot 48 (10 v/pc)

37° 8:31^{am} to 9:31^{am} Cold ✓

16 pc

M'113

RT

✓ 10 pc M'113 Not digest

✓ 1.6 pc 10x p95 lig. buffer

✓ 3 pc M'105 ~~Nhe~~ BsiWI ~~Nhe~~ oligo

✓ 1.5 pc T4 ligase m'77

18-15° 8:40^{am} to 11:53^{am}, RT 45° to 1pm, 37° 4pm to 1:28pm → Cold

Transformation

M'79 DH5α 80 pc ✓

(19) M'113 lig. 3 pc

0° 1:28 to 3:15 R, 20, 5, 20/16, 5/16

90 sec 42°

37° 1x Kan NZY

3:28 to whenever

V. Dry plates

TO SCOTH

If successful, use for excising

BspEI mitoCyan BsiWI

For Age I to put into Codetest.

T.H. lib. that. Scigo zapped - looks good.

Back from flying 172.5 in
NZ for 3 weeks !!

115

Sun Feb 2nd

Priority

Review of current status | mainly for AMHC constructs

#1

Check that into Cyan is as good as Cyan using

Bsp E1 into Cyan (Bsp WI) into (Age I) (Bsp WI)
will integrate well from M'113 (19)
PSKBI C/T pcr
K145 Age/Bsp

This has been zapped. Check status
in progress (See M'112)

#2

First step is M'14 | AMHC.NEO |

A N A C
MHC Neo AMHC Neo

check status → OK TH6-14 (24)

Sp I
↓
Aat II

and second step is

OK M'109 (8)

and excise

Aat II AMHC.NEO Not I OK TH6-18

for insertion into
Not I modified
M'33, BhrGN.AC
(20)

M'33 Aat II

Not I AMHC.Cyan
M'115 M33 Not I

M115 AN.AC

Remove
(BhrGN)
PSKBI

for later use to make animals & cells.

#3

A into C.AN

and

AC.AN

Xho
mlu

Hind
BspEI

Steps described on M'95.
check status

for
PSKBI, AVR II
vector

Mon. Feb 4 ch

Checked on mito Cym test
(mito CCTA)
TH 6-11

217.
seq #1 with
L'7 CT blue

218.
seq #1 with
K'17 Red Mlu

219.
seq #7 with
L'7 CT blue

220.
seq #7 with
K'17 Red Mlu

TH 6-11

Mon. Feb 4 ch

Checked on M'89 mini (18) see M'101

Tues. Feb. 6th

BspEI

Mto

Age I

On 11:12 am
11:43 am
29 Hx-1
20 100/100

Repeat PCR (n=20) for "mto"

① Dye
W
PRE-PCR
POST PCR
W
PCR
W
Dye

M'69 20x PCR buffer 25 μ l ✓
ddw 362 μ l ✓
M'69 DMSO 50 μ l ✓
M'69 dNTPs 2 μ l ✓
(1/20 μ g) 1/10 ~~PCR~~ PCR-Mto Vector 1 μ l ✓
10 M III Clontech
M'101 Bsp Mto Blue primer 20 μ l ✓
M'101 Red Cyan 2 primer 20 μ l ✓
480 μ l

20 μ l PRE M'119

to 5 tubes ~90 μ l each

On 11:15 am
11:19 am
29 Hx-1
20 100/100

② Dye W
W W
PCR
Post Bsp
M'119 B+A
892
33 M'121
W
PCR
W
Dye

Heat to 93° Add 4 μ l regular Taq to each
 $\geq 90^\circ$ 1 min 35 cycles
40 sec.
60° 8 min

On 4:20 pm Good start

Probably measure 40 cycles off ~?

Post! with 1/10 vol 8M NH₄Ac, 2.5 vol. EtOH

Back into 200 μ l as

M'119
PCR product

Take (60 μ l M'119 PCR product ✓
67 μ l 10x R1 salts ✓
+ 1 μ l (100) BspEI lot 3 (100 μ l)

37° ~4:15 pm to 5:35 pm

+ 3 μ l (60) Age I lot 34 (20 μ l) POST Bsp

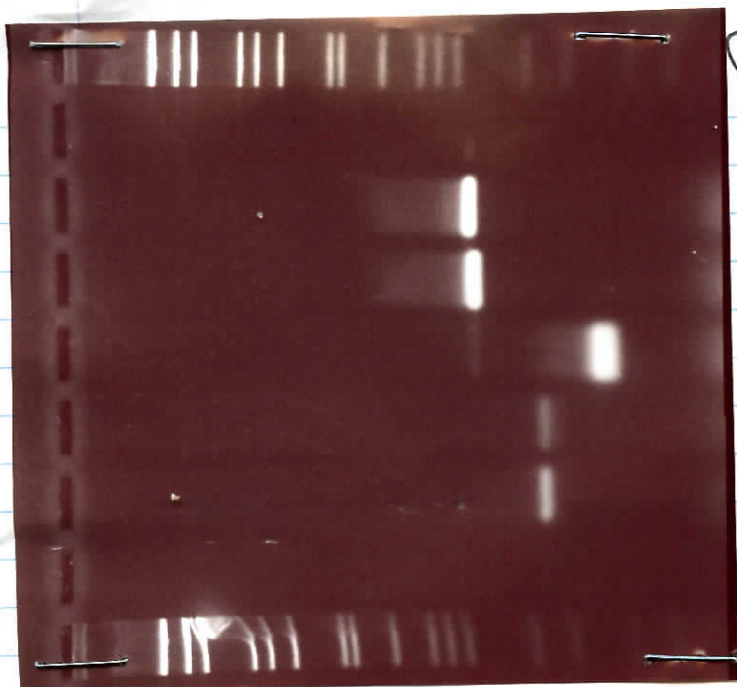
PRE Bsp

M'119
B+A

R.T. 5:35 pm to 7:15 pm

Need to inactivate Age I (BspEI does not matter)

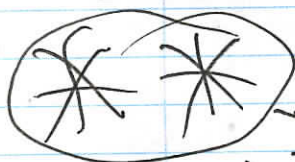
Freeze
→ ~80° for min.



Gel on M'18

Can use this PCR
for genotyping
Expected size - 154 bp
- is observed.

89
33



This M'89 Red Pool is dangerous.
It starts OK in BMHC and proceed
to the Age I site and then has a
deletion of ~250 bp to resume in hr GN.

225.seq check: 9026 from: 1 to: 40
/afs/isis.unc.edu/home/n/o/nobuyo/oliver/M33.BhrGN.AC check: 610 from: 8053
to: 25011

ASSEMBLE September 6, 2001 18:55
Gaps: 0 Quality: 280 Ratio: 7.368 Score: 26 Width: 3 Limits: +/-4

2 TTTACTTGNCGCCCAAGCTTCACCGGC TCGTGNAC 39
||||| |
8055 TTTACTTGTGCCCCAAGCTTCACCGGTTGCTAGCAC 8092

Not Age I

14 CCAAGCTT...CACCGGC TCGTGNACGAGCGCACCTGCGCTACGAGG 61
|| ||||| ||
8357 CCAGAGCTTCCCCGCGGCTTCGTATCAGAGCGCACCTGCGCTACGAGG 8406

62 ACGGCGGCTGGTGGAGATCCGAGCGACATCAACTGATCGAGGAGATG 111
||||| |
8407 ACGGCGGCTGGTGGAGATCCGAGCGACATCAACTGATCGAGGAGATG 8456

112 TTCGTGTACCGCGTGGAGTACAAGGGCGGAACTTCCGAGCGAGCGGCC 161
||||| |
8457 TTCGTGTACCGCGTGGAGTACAAGGGCGGAACTTCCGAGCGAGCGGCC 8506

162 CGTGATGAAGAAGACCATCAGCGGCTGCAGCCAGCTTCGAGGTGGTGT 211
||||| |
8507 CGTGATGAAGAAGACCATCAGCGGCTGCAGCCAGCTTCGAGGTGGTGT 2556

The original M'77 (4)
is almost certainly
incorrect.

32222 M'120 Red hr GN

CC CTC CAT GGT GAA
CAC GT

58%

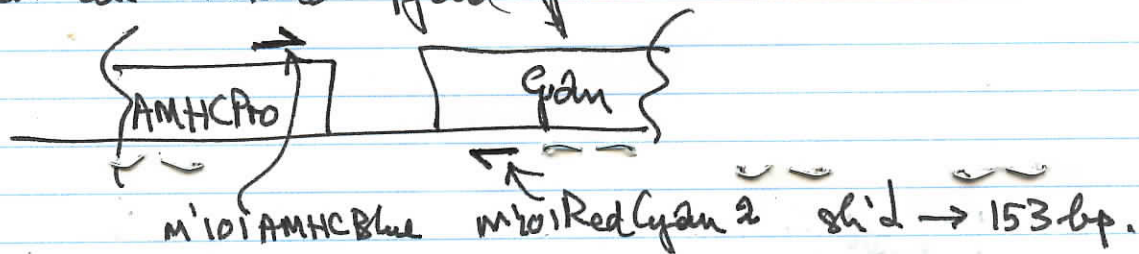
8183 M'91. Blue GN. A.mtoC

good sequence. Confirms
hr GN. ~~but not M'77~~
However, this sequence is
missing beginning of hr GN.
CARE! * * 5

Wed. Feb. 7th

Genotyping BhrGN.AC animals

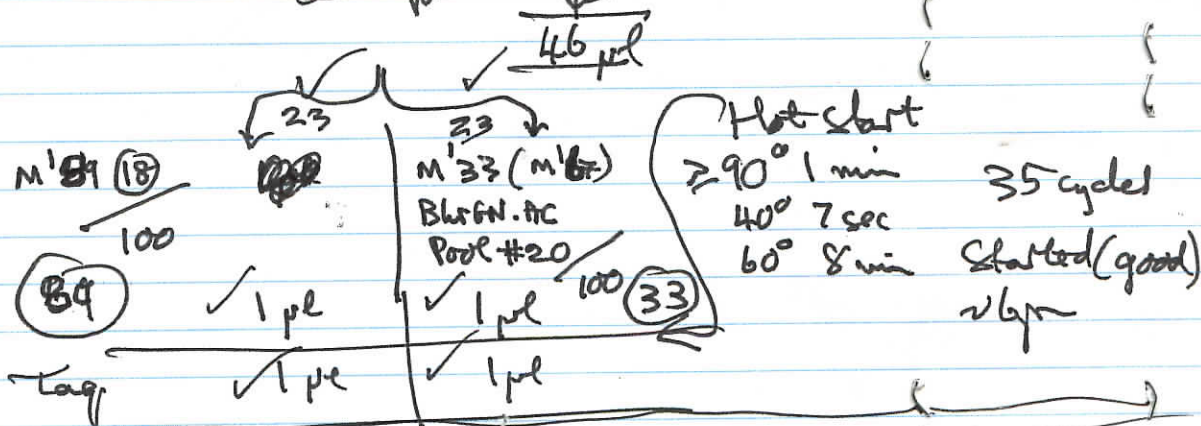
Two agouti female pups born to M'33 zapped chimera's.
Can use PCR to genotype: —



Take

$n = 2$

10x PCR 2.5 μ l ✓
 ddw 34 μ l ✓
 DMSO 5 μ l ✓
 dNTPs ≥ 0.2 μ l ✓
 M'101 Red Cyan 2 2 μ l ✓
 M'101 AMHC Blue 2 μ l ✓
~~10x PCR 2.5 μ l~~

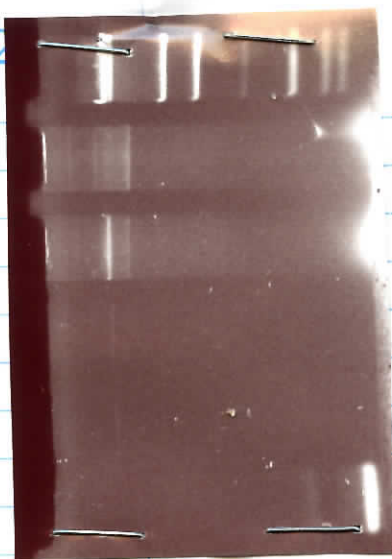


Another check on BhrG.AC single Age I

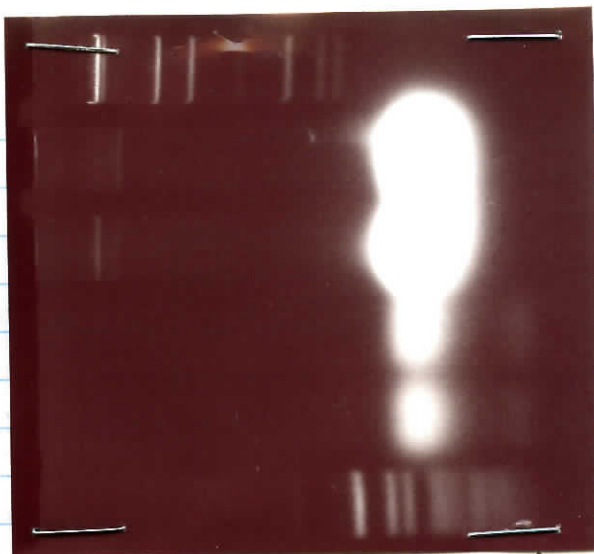
225. seq	L'93 Blue MHC /10 ✓ 5 μ l	M'89 Red Pool 1 μ l ✓	Water 14 μ l ✓
----------	-------------------------------	---------------------------	--------------------

T.H. 3-153
 formula used Blue BMHC 10
 Tried again here.
 Submitted Feb 11th 2002

12



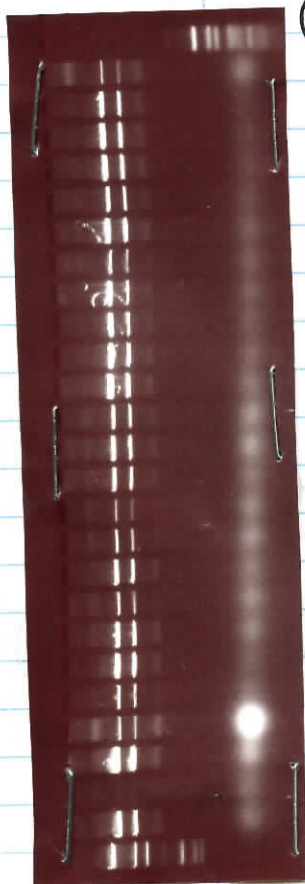
↑
Age Bln/AC



↑
Age Bln/AC

① Conclusion: Probably had better purify the Age I cut Bln/AC before ligation. Proceed but doubtfully useful.

② Conclusion. Not unexpectedly there are no inserts. Purify single cut & continue.



Mon Feb 11th 50 µl Bln/AC, 4.5 µl Lw, 25 µl (100 µl) BSS HII hot 13 (4 µl/µl)
 20 µl + 2 µl mini¹²³ ① - (2.4), 37° 12:00 to 1:10. Expect 2 kb if correct, but
 then need to find orientation.

✓	200 bp	200 bp
✓	2 kb	X
	9 kb	9 kb
	14 kb	16 kb

M'2:00pm
 14:50 Hx4
 0.8% 2v/cmm

Fri. Feb 8th

Age I

Age I

AGMM

Pre M'123

Post Age M'123

PRE his⁻ M'123

POST his⁻ M'123

ACR

W

Age

16 pl

M'123

PRE

Age I

ACR

①

R.T. 2:34^{pm} to 4:35^{pm}

+ 22 pl 5x CIAP buffer (1M Tris pH8)
 + 1 pl Lab CIAP

55° 4:41^{pm} to 5:15^{pm}

✓ 1x φOH / HQQ 2x CIAA

M'123
Age

16 pl
M'123
Post Age

M'123
Age CIAP
CIAA

ligation

Take ✓ 5 pl M'123 Age CIAP
 ✓ 3 pl M'119 B+A (check intact!)
 ✓ 18 pl ddw
 ✓ 3 pl 95% his⁻ buffer
 ✓ 2 pl M'72 T4 ligase

(BhrgN.AC)
(mito)

M'123
LIG

Cold 6:27^{pm} to 1:50^{pm}
 Fri Sat

M'123 Pre lig

M'123 Post lig

Transformation

1x AMP NZY

M'79 DH5α

85 pl

Rest 20, 5, 20, 5, 37° 6:20^{pm}
 16, 16 Sat

M'123 his⁻

8 pl

(None) 20 8 1 0

0° 5:30^{pm} to 5:50^{pm}

42° 90 sec

RT 1:50^{pm} Sun

(BSS HII will type)

M'123 ①-②4 1x AMP NZY

37° 4:30^{pm} 10 am Mon

226.seq check: 7209 from: 1 to: 797 /Reverse
/afs/isis1.bnl.edu/home/n/o/nobuyo/oliver/M92forM23 check: 1194 from: 4860
15817

ASSEMBLE December 25, 2001 14:13
Gaps: 2 Quality: 7792 Ratio: 9.801 Score: 796 Width: 11 Limits: +/-12

797 CTTCCTTCCTTCCTTCCTTCCTGGGTCAGAGTGCTGAGAATCACACCTGG 748

4862 cttccttccttccttccttcctgtgtcagagtgtctgagaatcacacctgg 4911

747 GGTTCACCCCTTATGTAANCAATCTTCCAGTGAGCCACAGCTTCAGTGC 698

4912 gattccacccttatgtaaatctctcagtagccacagcttcagtc 4961

697 TGCTGGGTGCTCTTAAGCTTCTCAGCCCTGGCTGTGCTCTGTTCATC 648

4962 tactgaagatgctctctaccttctcacccttgcttgctctgttccatc 5011

647 CTGGTCAGGATCTCTAGATTGGTCTCCACCTCTCTACTCCTCTTCT 598

|||||
5012 ctggtcaggatctctagattggctcccgacctctgctactcctcttct 5061

597 GCCTGTTCTCTCTGTCCAGCTGCGCCACTGTGGTGCCTCGTTCAGC 548

5062 g c c t g t t c c t c t c t g t c c a g c t g c g c c a c t g t g g t g c c t c g t t c c a g c 5111

10:17 PM OK AM

7C/2-27 Order Am
-t. Co.

(21) mitolofon

247 GACAGACAGATCCCTCTATCTCCCCATAAGAGTTTGAGTCGAACCGTC 198
|||||

5412 gacagacagatccctctctatctccccataagagtttgagTCGAACGTC 546

197 AGATCCGCTAGCATGTCCTGACGCCGCTGCTGCTGCCGGGCTTGAC 148
|||||

5462 AGATCCGCTAGCATGTCCGTCTGACCTCCCTGCTGCTGCGGGGCTTGAC 551

147 AGGCTCGGCCCGGGCGGCTCCAGTGC CGCGCGCCAAGATCCATTCGTTGG 98
|||||

5512 AGGCTCGGCCCGCGGCTCCAGTGCCGCGCGCCAAGATCCATTCGTTGG 556

97 GGGATCCACCGGTGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTACC 48
|||||

5562 GGGATCCACCGGTGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTACC 561

47 GGGTGGTGGCCATNCTGTCGAGTGGACGGGGNACGTAACGGC 1
|||||||

5612 GGGGTGGTGCCTCACTCTGGTGGAGCTGGACGGCG.ACGTAAACGGC 5656

226.seq 0 check: 7209 from: 1 to: 797 /Reverse

277 GAGTCCTGGTGGGAGAGCCATAGGCTACGGTGTAAAAGAGGCAGGGAAGT 228

11872 gagtcctgggtggagagagccataggctacgggtgtaagagaggcaggggaagt 119

227 GGTGGTGTAGTAAGTGGGACTTCACATAGAAGCCTAGCCACACCAGA 178

11922 ggtggtgtaggaaagtcaggacttcacatagaagcctagccacaccaga 119

177 AATGACAGACAGATCCCTCCTATCTCCCCATAAGAGTTTGAGTCGACCT 128

11972 aatgacagacagatccctctatctccccataagagtttgagtcgacct 1202

127 CTGCAGCCAATATGGGATCGGCCATTGAACAAGATGGATTGCACGCAGGT 78

12022 ctgcagccaatatgggatcgggcattgaacagatggattgcacgcaggt 1207

77 TCTCCGGCCGCTTGGGTGGAGAGCTATTGGGCTATGACTGGGCACAACA 28

|||||
12072 tctccggccgcttgggtgggaggctattcggctatgactgggcacaaca 1212

27 GACAATCGGCTGNTGGTGATGCCGCCG 1

12122 gacaatcggctgct.ctgatgccgccg 12147

```
227.seq          check: 1312 from: 1      to: 805    /Reve
/afs/isis.unc.edu/home/n/o/nobuyo/oliver/M92forM23 check: 1194 from
```

mol Red Cyan 2.
226. sep conforms

AMHC PRO - into Cyan
border

See also

N'83

M'125 Red Neo
227. seq confirmed
AMHC PRO - Neo
border

Sun. Feb 10th

Prep gel for Age I (18) ~~similar~~

Take $\checkmark$ 40 μ l M'89 Red Pool
 $\checkmark$ 95 μ l ddw
 $\checkmark$ 15 μ l 10X RI salts
 $\checkmark$ 5 μ l (100) Age I hot 24 (20/ μ l)
 RT 12:03^{pm} to 3:25^{pm}
 M'125 PRE

$\checkmark$ + 35 μ l IM Tris pH 8 (5X CIAP buffer)
 $\checkmark$ + 3 μ l hot CIAP
 50-55 $^{\circ}$ 3:26^{pm} to 4:25^{pm} 1x ϕ OH/HO ϕ , 2x CIAP

CANCEL. See M'120 M'125 Age CIAP¹⁸

Tues. Feb 12th

Sequence checks on J.C. M92 for M23

GGG CGC CCC TGC GCT GA

M'125 Red Neo

Submitted Feb 12th near Kas I -- Bbe I region.

Also use M'101 Red r n. 9

	M'101 Red Cyan 2	Jc6-27 (21)	Water	M'125 Red Neo				
				GGG	CGC	CCC	TGC	GCT
226. seq	10 5 μ l	1 μ l	14 μ l	17 82%				
227. seq	M'125 Red Neo 10 5 μ l	Jc6-27 (21) 1 μ l M'125	Water 14 μ l					

Submitted Feb 13th

~~72268~~ Oligo Name: M'126 ~~Spe~~ Asc ~~Spe~~
~~14~~

C	T	A

G	A	G

G	C	G

C	G	C

C	T

~~72269~~ Oligo Name: M'126 ~~Not~~ Asc ~~Not~~
~~868~~

G	G	C

C	A	G

G	C	G

C	G	C

C	T

Mlu I
 A CGCG T

Tues. Feb. 12th

Forget about M'97 scheme!

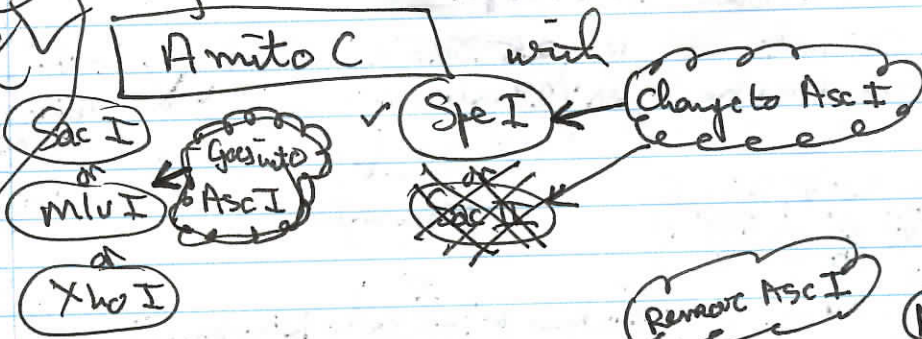
~~POSTPONED~~
~~CHANCE~~

New Start on

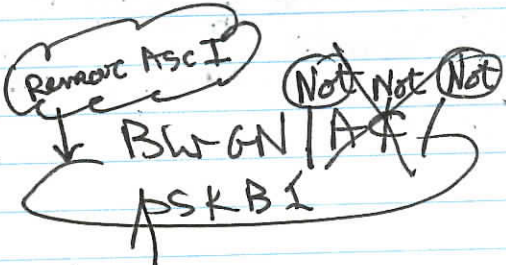
Bhr GN. A mito C

PSKB1

Using M92 for M23 can excise fragment



Can remove AC from after removing Asc I



with Not I and change to Asc I

Can cut out Bhr GN from Bhr GN. AC
~~ACT II~~ Not I

Steps would be: -

- (i) change ~~Sac I~~ Spe I to Asc I in M92 for M23 and excise

Mlu I | A mito C | pLA Spe Asc I
- (ii) remove Asc I from Bhr GN. AC PSKB1, (M'33)
 (one linearization site)
- (iii) remove AC with Not I & change Not to Asc I

Asc / Mlu

Asc
- (iv) assemble Bhr GN / A mito C PSKB1 from (i) and (iii)

M'128/33
 Transformation
 37° 2:07 pm
 1x NZY, 1x AMP R 20 5 20 5
 16 16
 M'128/33 (4) (0) (16) (16)
 Minis M'128/33 (1)-(4) Blue 37° 12:20 pm Fri
 M'128 mito
 Transformation
 37° 5:20 pm
 1x NZY, 1x AMP R 20 5 20 5
 16 16
 M'128 mito (12) (3) (0) (0) (0)
 Minis (5)-(24) Red M'128 mito 37° 12:20 pm Fri
 12.13 DH5α 80 pl ✓
 M'129 M33 lig 4 pl ✓
 0° 1:33 2:05 pm
 90 sec 42°
 M'128
 2/12 DH5α 80 pl ✓
 4 pl ✓ M'131
 Red M33 lig
 (mito)
 M'128²
 2/12 DH5α 80 pl ✓
 12:50 pm
 Fri Blue
 12:20 pm
 don Set
 (Cody)



Conclusion: M'33 Asc is
 good. AutoC spe has
 rather a lot of single
 strand DNA so may be
 a problem. Revised.

Transformations were poor; repeat with new DH5α
 and also with more M'128/33 ligase.

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89 μ g
1
gⁿ
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100

Thurs. Feb 14th 05

AXI
BLG-N. AC.
M3305

M'127 step (ii)

PCR
PRE Asc {M'33
POST Asc/T4 (20)
PRE Spe {Amuto C/AN
POST Spe {AN
AG
W
Page

Take 5 μ l ~~PCR~~ M'33 (20) Pad
10 μ l 10x NEB buffer 4
85 μ l ddw
1 μ l (10 u) Asc I lot 10 (10 u/ μ l)
37° 10^{am} to 11:40^{am}

M'129
Asc/T4

+ 9.5 μ l 10x T4 DNA polymerase buffer (NEB)
+ 3.7 μ l 25 mM (4) dNTPs for 1 mM final
+ 1 μ l (30) T4 DNA polymerase lot 33 (30/ μ l)
37° 30 min 11:45^{am} to 12:15^{pm}

M'129
M33 high

Take 2 μ l M'129 Asc/T4
15 μ l ddw
2.2 μ l 10x p95 ligase M'77:20
3 μ l T4
37° 12^{to} 1:30^{pm}

Post Asc/T4
M'129

Spe → Asc I

M'127 step (i)

Amuto C/AN

M92 for M23

Take 3 μ l JC6-27 (21) M'125
6 μ l 10x RI salts
51 μ l ddw
1 μ l (10 u) Spe I lot 28 (10 u/ μ l)

M'129
Spe

37° 11:06^{am} to 12:26^{pm} 16 μ l Post Spe M'129

16 μ l
PRE Spe
M'129

Take 4 μ l M'129 Spe digest
+ 2 μ l p95 10x buffer
+ 4 μ l M'126 Spe Asc Spe oligo
+ 2 μ l T4 ligase M'77
+ 8 μ l ddw
Cold 12:30^{pm} Thurs - 1:30^{pm} 4:40^{pm}

M'129
mito C
high

45° 3:40^{pm} → RT 3:40^{pm} → 4:40^{pm}

Fri - Feb. 15th

Repeat M33 ~~As~~ ligation & back transform^w

Take 4 ~~pl~~ M' 129 Asc/T4

✓ 4 ~~pl~~ ddw

✓ 1 pl p95 10x ligation buffer

✓ 1 pl T4 ligase M' 77

37° 9:18 to 11:20am (cold)

M' 131
M33 lig^w

↳ x of previous
(see M' 128)

Back Up Transformation (M' 128)

Plated onto (very dry) 1x AMP 1x NZY plates 37° 2:45 pm Fri

Blue R, 20, 5, 20/16, 5/16 M' 128/33 repeat

Red R, 20, 5, 20/16, 5/16 ~~mito~~ M' 131 ligation

Sat. Feb 16th

Better
M' 97

Alternative to BhGN.A.mitoC
route PSKB1

Xma Fragments:-

Start End
9.3 9.3
6.6 (4.7)
1.9

(i) Start with M92 for M23. Change SpeI to XmaI
→ excise

A MHC ~~mito~~ CpeA

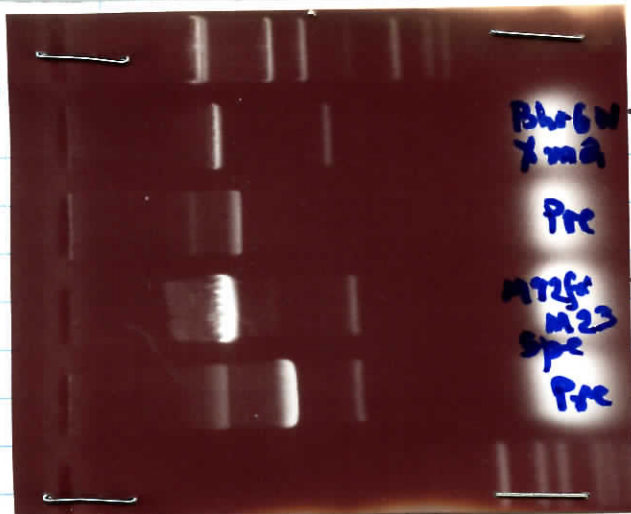
biggest M' 33

(ii) with Xma & CIAP
Do not need to purify.

BhGN.A.C. junk fl

PSKB1

Then ~~excise~~ replace insert(s) with above. Check orientation;
with Age I



Revised Photo
 (1) Conclusion: Cuts are fine.
 Two Xma sites on M92 for M23 →
 sizes are 5.5 kb + 19.6 kb - checks
 Bhr6W
 Xma

4.5 kb
 19.6 kb
 5.5 kb

M92 for M23
 Spe

M133 POOL

(2) 2, 3, 9, 10, 11, 12

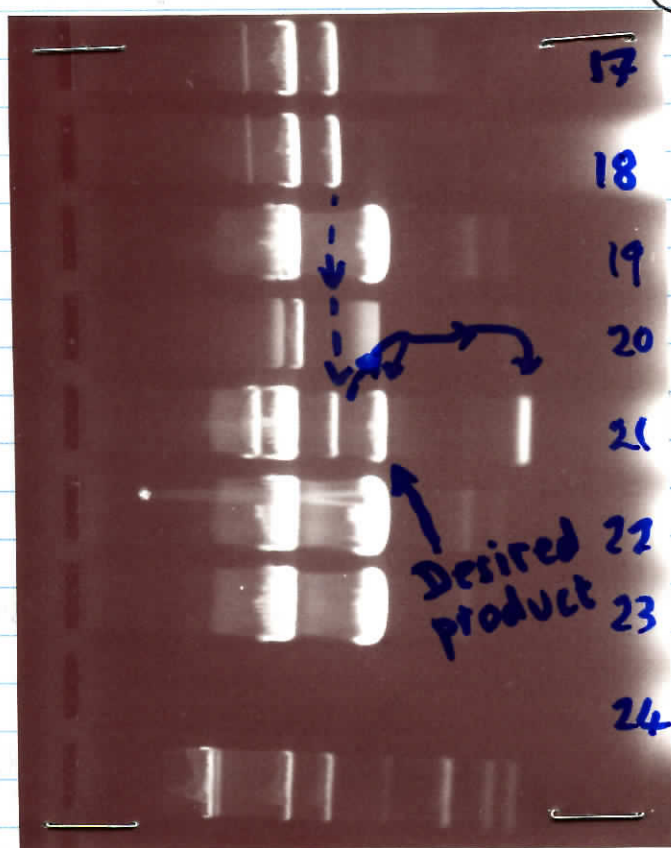
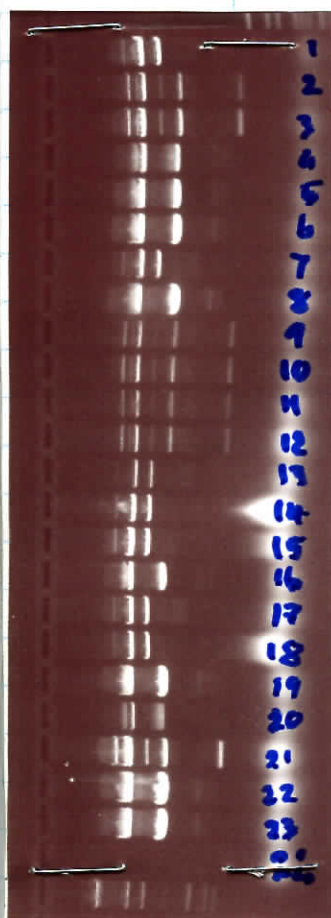
2 & 21 are correct
 (1.9, 4.7, 9.3)

1, 7, 13, 14, 15, 17
 2 & 18 are initial
 plasmid (6.6 + 9.3)
 Proceed with

#21
 M133

as M925 to K
 pe ma

(1) Agg
 W
 PCR
 (1) 5-20
 M133
 Xma
 (2) Agg
 W
 Dyl



Desired
 product

Take ✓ 50 µl 10x NEB buffer 4

✓ 50 µl 10x Acl

✓ 396 µl H₂O

✓ 7.5 µl (75) XmaI lot 60 (100/µl)

1 µl M1335 → X minus + 20 µl 37° 2:46 PM 3:46 PM

30/RX

Bottom
 Plate
 R,
 Mo
 1x

Sect - Feb. 16 d

3:25
4:05
0.26 HXL
M92

Dye ①

W ACR Step (i) M'131

PRE } M'133
POST }
AG- PRE M'133
W POST Xma
Dye

M'125 JCB-27 ② M92 for M23
Take 3 μ l
6 μ l 10x RI salt
50 μ l ddw
1 μ l (10u) Spe I Lot 28 (10u/ μ l)

37° 8:50 am - 1:20 pm 10 min $\leq 80^\circ$ 16 μ l
PRE Spe M'133
M'133 M92 for M23
15 μ l M'133 Spe
4 μ l M'97 Xma Spe
2 μ l M'77 T4 ligase
2.3 μ l p95 10x ligase
15° 1:40 pm BT 3:10 37° 3:48
POST Spe M'133
4:13 to cold

Step (ii) M'131
Take 10 μ l M'33 Bhr GN.AC Pool #20
20 μ l 10x NEB buffer k
20 μ l 10x Alb-m133
150 μ l ddw
5 μ l (50u) Xma I Lot-60 (10u/ μ l)
37° 2:17 to 3:20 pm $\rightarrow$ 4:50 Frozen
10 min $\leq 80^\circ$ Heat shock!
Bhr GN.AC
Sum + 4.4 μ l M Tris pH 8
+ 2 μ l Lab CIAA
55° 11:24 12 pm 1x ϕ OH/HOQ, 2x CIAA
Can use this directly without purification.

Better dated

Plated 2x AmpNZY

R, 20, 5, 20, 5 37° 4:47 pm 90 sec 42°
Manning ① ① ①
11 am Sun

1x AMPNZY mini M'133 RT 11:15 am Sun
S $\rightarrow$ X 37° 3:43 pm - 9 am Mon

80 μ l
5 μ l
M'133
S $\rightarrow$ X
M92 for M23

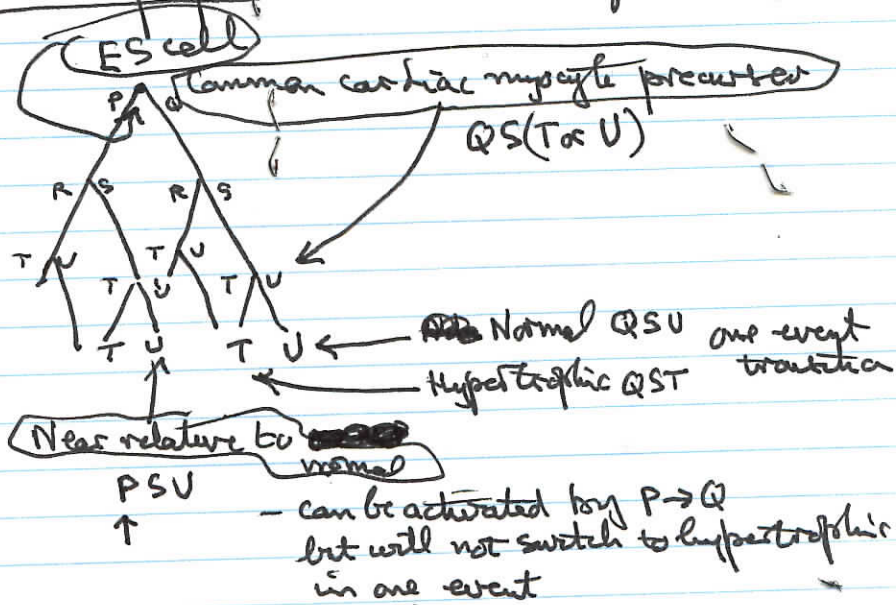
Purify this fragment

Sun. Feb. 17th

Logic of Adult Normal $\Rightarrow$ Fetal Hypertrophic

Since many different factors, including transgene overexpression of a MAPK kinase, can stimulate N to H (normal to hypertrophic), I need to find common factors that prevent all of these switches. This would identify elements that are essential to the transition, $\rightarrow$ could be used to develop preventative drugs.

Some other thoughts on alternate states P vs Q, R vs S etc.

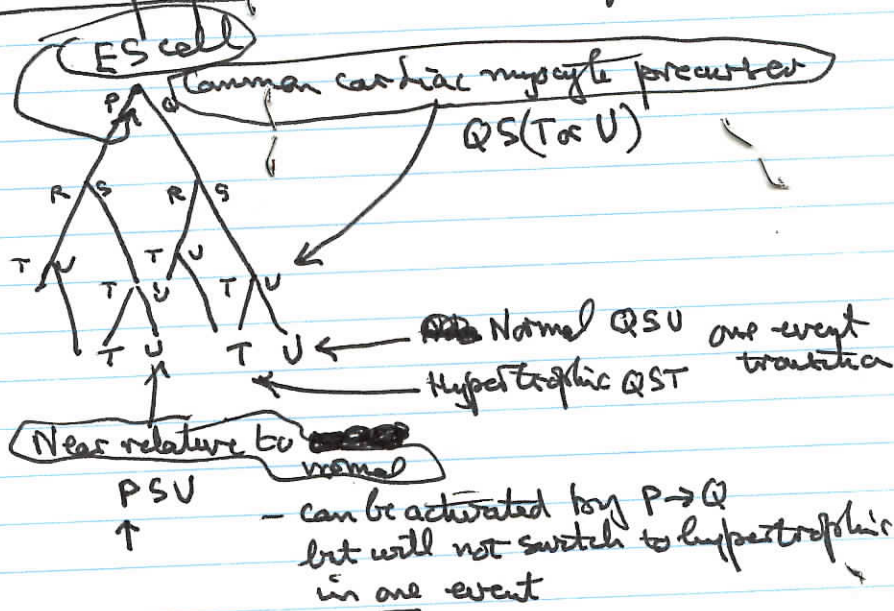


Sun. Feb. 17th

Logic of Adult Normal $\rightleftharpoons$ Fetal Hypertrophic

Since many different factors, including transgene overexpression of a MAPK kinase, can stimulate N to H (normal to hypertrophic), I need to find common factors that prevent all of these switches. This would identify elements that are essential to the transition, & could be used to develop preventative drugs.

Some other thoughts on alternate states P vs Q, R vs S etc.



A dark, high-contrast image showing a vertical strip of light reflecting off a textured surface, possibly a book cover or a document page. The image is rotated 90 degrees clockwise.

② Good fragment

(2) On 2:14 PM
Off 3:10 PM
Duty W
W
W
W
W
W
W
W

0.8
HxL
max

agt

8

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सु

Xma

Xma

Mon. Feb 18th

Isolation of Xma

AMHC PRO into C / AMHC Neo

Dye ①
AGMM

~1386
M'137
Xma 21

AGMM
Dye

- Take ✓ 20 μ l 10x NEB buffer 4
- ✓ 20 μ l 10x Alb m'133
- ✓ 147 μ l d₂W
- ✓ 10 μ l M'133 mini (21)
- ✓ 3 μ l (30U) Xma I lot 60 (10U/ μ l)

M'137
Xma
21

37° 6:25 to 8:50 pm

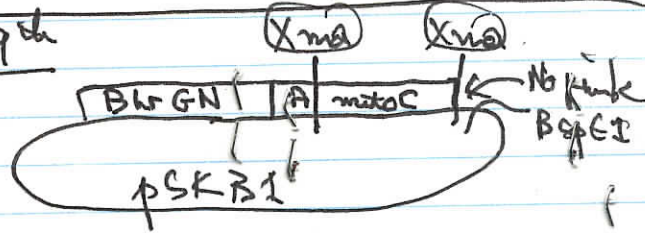
Use Marker in place of RNAse.
Recovered into 80 μ l as

M'137 AmtoC Xma frag

Tues. Feb 19th

Ligation

Take μ l M'133 Xma
CIAP CIAA



Unable to find this! ⚡! Most unusual!!

Repeat M'133 protocol

- Take ✓ 10 μ l M'133 BhGN.AC Pool #20
- ✓ 20 μ l 10x NEB buffer 4
- ✓ 20 μ l 10x Alb
- ✓ 1450 μ l d₂W
- ✓ 5 μ l (50U) Xma I lot 60 (10U/ μ l)
- 37° 2nd to 3:02 pm

M'137
BhGN.AC
Xma
CIAP
CIAA

16 μ l M'137 Post Xma

+ 40 μ l 1M Tris pH 8

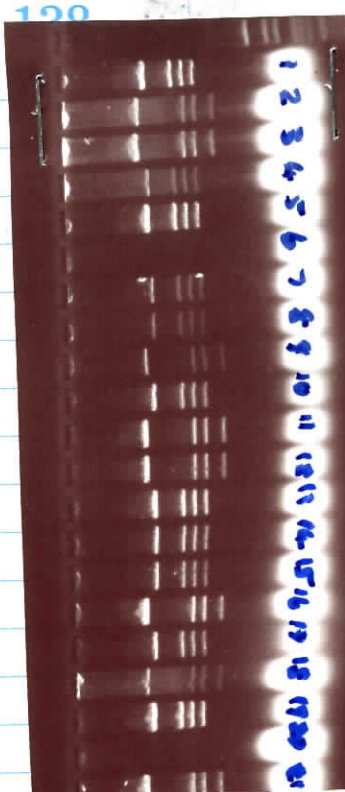
+ 2 μ l l₂ CIAP frozen
55° 3:10 pm to 3:38 pm

Sample found!!

Ligation truncated

- Take ✓ 10 μ l M'133 Xma CIAP/CIAA
- ✓ 2 μ l M'137 AmtoC Xma frag
- ✓ 15 μ l 10x p95 lig. buffer
- ✓ 1.5 μ l M'77 T4 ligase
- 15° 3:50 pm ✓
- RT 4:50 pm ✓
- 37° 5:15 pm ✓
- 5:35 pm Cold

M'137
BhGN.AC
BhGN



Conclusion ①

- ⑫ 2, 3, 9, 11, 12, 16, 18, 21 appear to be mito Rev
- 17 1, 4, 5, 7, 8, 10, 13, 14, 15, 17, 19
- 18 appear to be correct 22 look like BhrGN.AC.
- ⑬ Check by sequencing.
- 20 6, 20, 23 & 24 are v. low
- 21 M'139 Blue Pore is, presumably 1, 4, 5, 7, 8 etc.
- 22 M'139 ⑬
- 23 is BhrGN.AmitoC
- 24 pSKBI

231.seq check: 8340 from: 1 to: 850 /R
/afs/isis.unc.edu/home/n/o/nobuyo/oliver/M131.BhrGN.AmitoC check:
916 to: 24273
ASSEMBLE February 17, 2002 12:39
Gaps: 9 Quality: 6993 Ratio: 8.425 Score: 653 Width: 21 Lim, 2002 12:39

check: 1893 from: 1 to: 848
/o/nobuyo/oliver/M131.BhrGN.AmitoC check: 2417 from: 1 to: 872 /Reverse
er/M33.BhrGN.AC check: 610 from: 15633

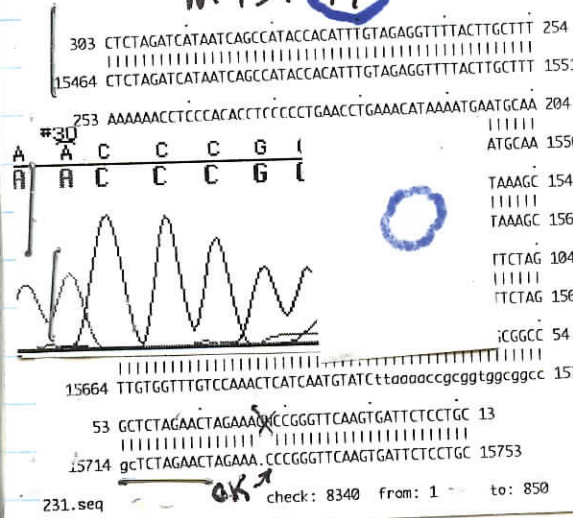
842 CANACTAAGTGGGAGAGT.....NNAGCGHNNNGCGGCAANGAA 803
14921 CACCTGACCTGGGCGTGCAGTGCTTCAAGTCCGCGTACCCGACCATGA 14970
802 NGANNCAANAATTCTNNNGTCCGATNGCCNGNGGNT.NGTNCAGHAG 754
14971 AGCAGCAGGACTTCTTCAAGTCCGCGTACCCGAGGCTACGTCCAGGAG 15020
753 NGCANCATNTTNNHANGAGAGCGGCACTNCAAGACNGCGCCNAGG 704
15021 CGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCC.GAGG 15069
703 TGAAGTTNAGGGCGACACCTCTGGTGAACCCGATCGAGCTGAAAGGG 654
15070 TGAAGTTGAGGGC.GACACC.CTGGTGAA.CCGCATCGAGCTGAA..GG 15114
653 GCATCGACTTCAAGGAGGACGGCAAACTCTGGGACAAAGCTGGAGTA 604
15115 GCATCGACTTCAAGGAGGACGGC.AACATCTGGGACAAAGCTGGAGTA 15163

Score: 577 Width: 43 Limits: +/-44
ANGCTTNTAAGANCNHAG 821
GTAAACTCTTATGGCAGTA 15692
GTGGNGAGAGNAGGAATATG 771
TTTTTCTTGAGACAGGATATT 15742
NANANNNTNGCCNATGTA 721
GCACAGTCTTGGCTCACTGCA 15791
NCCCCGCCACGCTCCCAAG 671
CTCCACCTCAGCTCCCA. 15838
AGGCANATAATTTTTATT 621
AGGC.CAGATAATTTTATT 15887

M'139 ⑬

39 ⑫

22



303 CTCTAGATCATAATCAGCATACCATTTGTAGAGGTTTACTTGCTTT 254
15464 CTCTAGATCATAATCAGCATACCATTTGTAGAGGTTTACTTGCTTT 15513
253 AAAAAACCTCCCAACCTTCCCTGAACCTGAAACATAAAATGAATGCA 204
ATGCAA 15563
TAAAGC 154
TAAAGC 15613
TTCTAG 104
TTCTAG 15663
ICGGCC 54
15664 TTGTGGTTGTGCCAACTCATATGATCttaaaccggtggcgccc 15713
53 GCTCTAGAACTAGAAA.CCGGGTTCAAGTGATTCTCCTGC 13
15714 GCTCTAGAACTAGAAA.CCGGGTTCAAGTGATTCTCCTGC 15753

Score: 577 Width: 43 Limits: +/-44
GTGCATATATATACATACA 221
GTGCATATATATACATACA 16286
CAAAATGATCCTGTTTATGTA 171
CAAAATGATCCTGTTTATGTA 16336
TTTTATTTATTTTATTTT 121
TTTTATTTATTTTATTTT 16386
TCGCGGAGGCTGAAGTGCAGT 71
TCGCGGAGGCTGAAGTGCAGT 16435
GCCGNCGGGTTCAAGTGATT 21
GCCTCCCGGTTCAAGTGATT 16484

OK

0.876HX4 ON 12:31pm OFF 1:33m

M131. Bhr GN. AmtoC

139

Bye ①
PCR
① } Bam HI
① } M'139
② } minis
Bhr GN. AmtoC (card)
AG
W
Bye

Tues. Feb 19th

Bhr GN. AmtoC / Bsp

PSK B1

Transformant

DH5 2 M'128 80 µl

M'137
Bhr GN. AmtoC 5 µl

0° 5:36 to 5:55

42° 90 sec.

2 XAMP NZF
R, 20, 5, 20, 5, 0
16, 16

37° 6:03 pm

Picked RT 12 noon Villam

37° 2 pm (Wed) (Thurs)

Can orient & check nits using Age I.

Bhr GN. AC → 8078 14610

Bhr GN. AmtoC → 8078 14720

Bhr GN. A reversed nitoC → 8078 12073

6.4 at Bam HI 6.4
680 4540 9100 9170 15610
10.3 680 4540 9100 9170 14715
12.9 680 4540 9100 9170 12090
3.9 4.6 70

Thurs. Feb 21st

Take 50 µl 10X RI salts

✓ 448 µl dH₂O

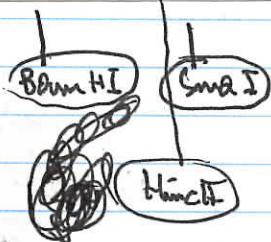
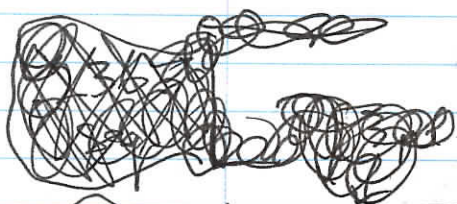
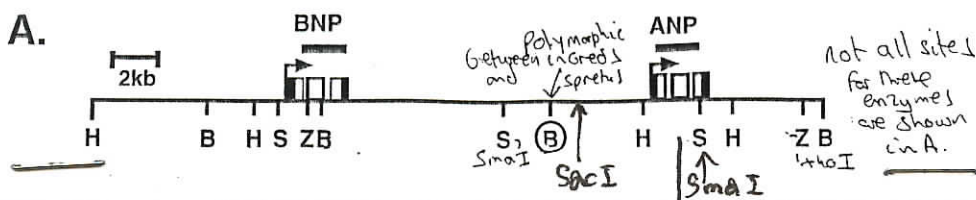
✓ 2.5 µl (500) Bam HI hot 82 (200/µl)

2 µl of M'139 + 20 µl 37° 11:17 to 12:20

230. seq	L'121 Red Arr 10 5 µl	M'139 (16) 1 µl	Water 14 µl	Confirming this is M'131 Bhr GN. AmtoC correct
231. seq	L'121 Red Arr 10 5 µl	M'139 (19) 1 µl	Water 14 µl	Confirming this is M'131 Bhr GN. AmtoC correct
232. seq	L'121 Red Arr 10 5 µl	M'139 (22) 1 µl	Water 14 µl	Confirming this is M'131 Bhr GN. AC

L'121 Red Arr is 3' to Bsp E1

86 and *M. Spretus* genomic structure



233. seq

M'140 PNT ANF3
SJ4-157
20.2 pg/pc
1 pl

M'140 PNT Not Blue
10
5 pl

Water
14 pl

No sequence primer
Remain primer
without ~~sequence~~

234. seq

M'140 PNT ANF3
1 pl

M'140 PNT Bam Blue
10
5 pl

Water
14 pl

Submitted to
Lab 26

235. seq

M'140 PNT ANF3
1 pl

M'140 PNT Xho Red
10
5 pl

Water
14 pl

Confirm
expected sequence
into Hinc II in both
directions.

Can use three PNT-specific primers to check the important junctions in human construct :-

M'1111 PNT Not Blue

734 7348 11 8
CGCGGTG GCGGCCGC
1826 1826 1826 1826
AAGCTGACTCTAGAGGATCC
22 22 22 22
CTGCRGGGGCCCTCGAG 8

1845 PNT numbers

32357
15
98%

M'140 PNT Not Blue					
C G C	G G T	G G C	G G C	C G C	

32358
20
50%

M'140 PNT Bam Blue					
A A G	C T G	A C T	C T A	G A G	
G A T	C C				

32359
7

M'140 PNT Xho Red					
C T G	C A G	G G G	C C C	T C G	
A G					

3

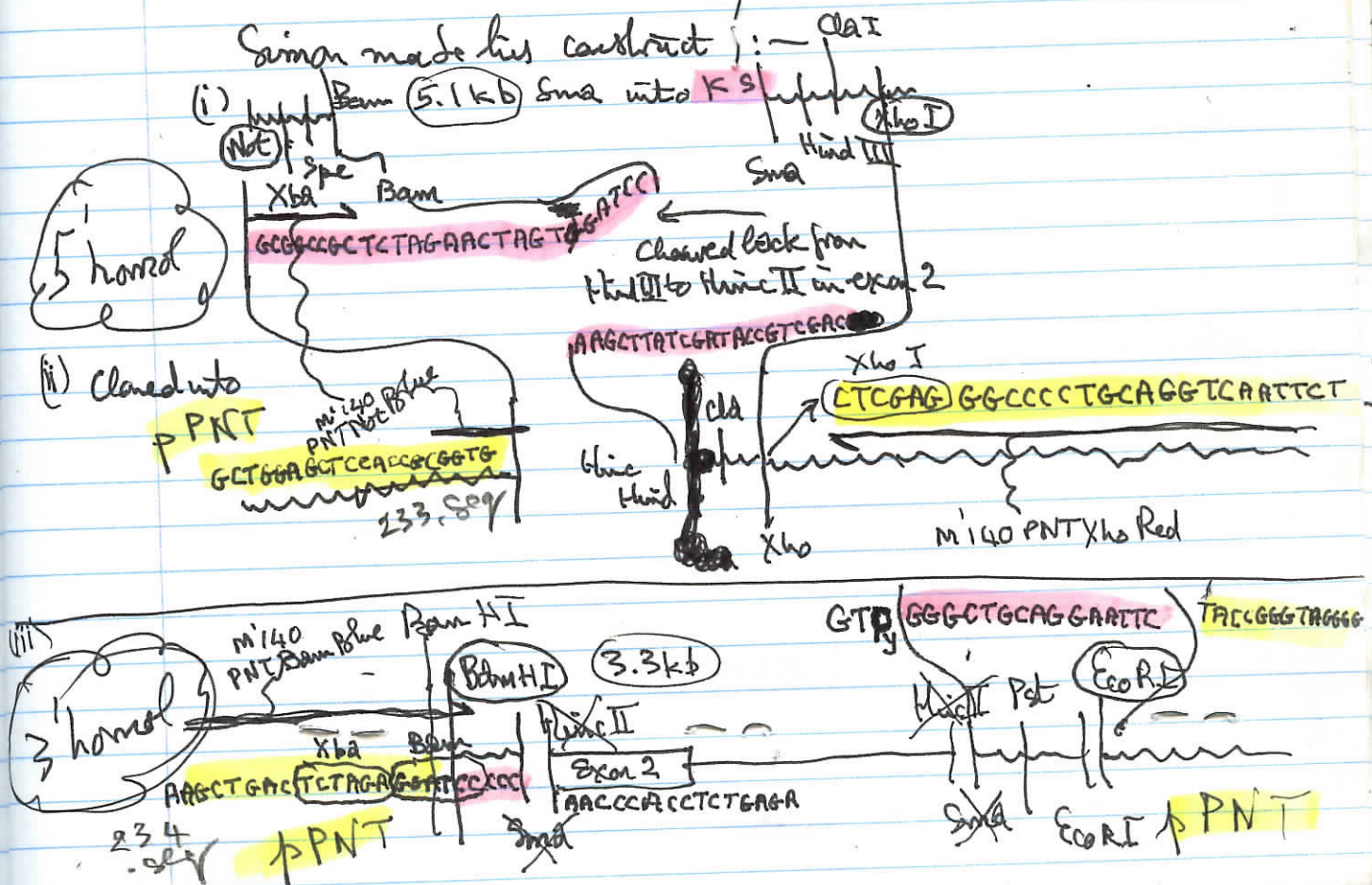
Wed. Feb. 20th

Backup for Exon 3 TG^R targeting

Since we have not been doing well in using exon 3 vector (M'22) for TG^R targeting, I need to have an alternative. Best bet seems to be to use O-type recombination to insert AnatoC. AN between ~~exon 3~~ ANP & p10 BNP. The map from Simon's lab is an M'140 & the (Bam) ANP (Bam) clone is available. Would be easier if I had ~~the~~ upstream of the Bam HI clone. Or could use Ω with current Bam fragment. As a start it will be useful to sequence the Bam $\rightarrow$ Sac $\rightarrow$ region of Simon's targeting construct.

Sat. Feb. 23rd

Simon made his construct :-



Feb 22nd, 2002.

Sorted cells by green signal

Construct name: β MHC.hrGN + α MHC.Cyan

Bright field



Yellow filter



Green filter



One day after sorting

Object: x20

Exposure time: 0.5 sec.

Source: Differentiating cells from
ES to beating 10 days after plating

Red filter



Cyan filter



(same cells are beating)

OS/NM 11th
contract
renewal
date.

143

Fr. Feb 22nd 2002

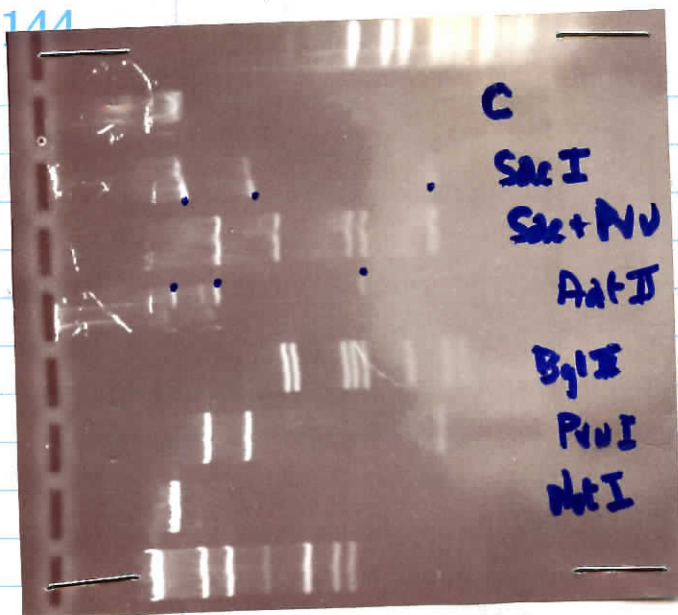
ES cells were dispersed & allowed to
form clasts in liquid culture for 2 days in P100
medium. Whole of culture was plated ~~into 6 wells~~
~~into 6 wells~~ into 6 wells of (laminin coated) $t=0$

At $t=10$ days, 5 wells PBS+EDTA 10 min.
& then PBS+trypsin + EDTA for 30 min. Pipette
filter (70 μ m) & set filtrate using green fluorescence.
One day later ($t=11$), photographs were taken.

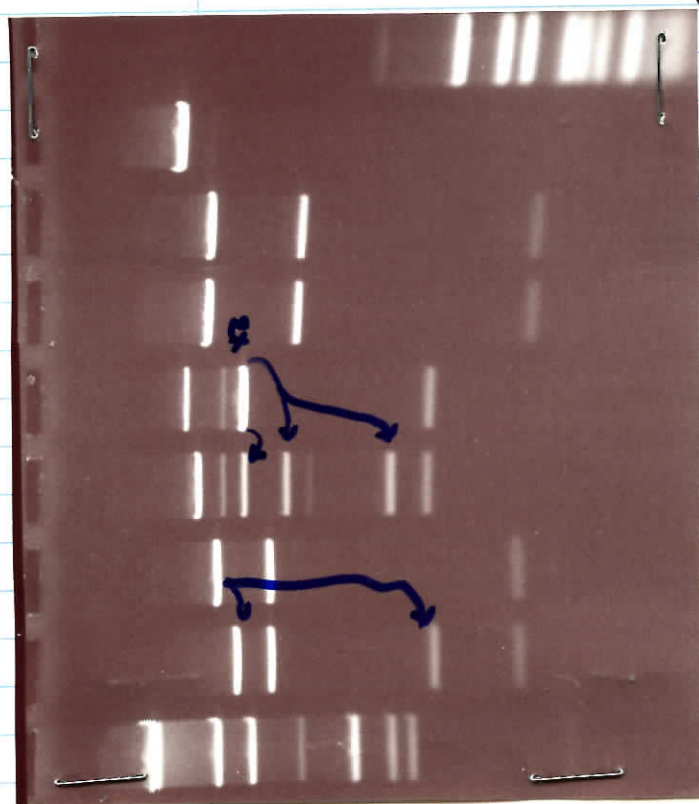
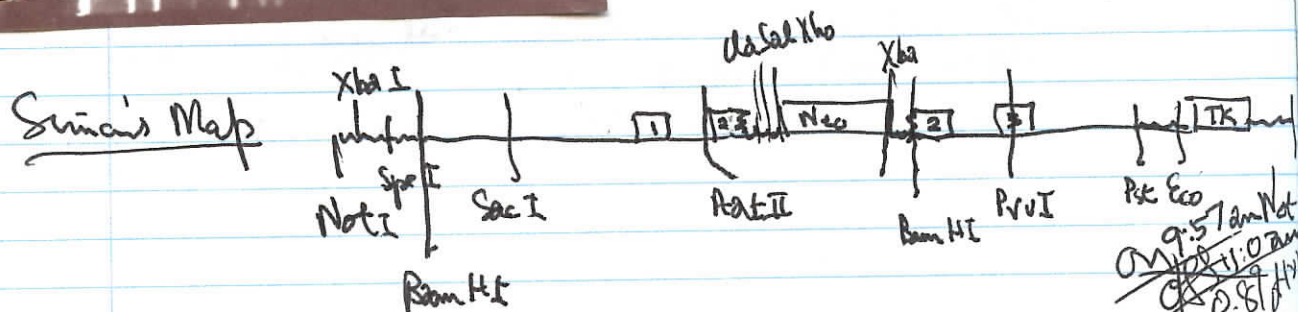
Alive but not yet beating

Note one myocyte has typical morphology
(bottom left) and is blue.

Notes, cells that attached spread out & began to beat again.



① Conclusion: A bad gel! but even so there are at least 3 sites for each enzyme except Not I. Wait for new restriction plasmid before proceeding. Except by Sac I, Aat II and Pvu I ± Not to identify the Not end.



② Conclusion. Some of double digests were incompatible with Not I. Cannot see Not I action with Sac I or else they are very close together. Sequence will tell. The ~~Not~~ Pvu piece Not

is probably good. Aat II cuts in position 1; hadn't noticed this.

Not
Xba
Hind
Dye
w
ACR
cont
Sac
Sac
Bgl
Not
AC
w
Dye
1

9.57 am Not
11.02 am
0.81 am

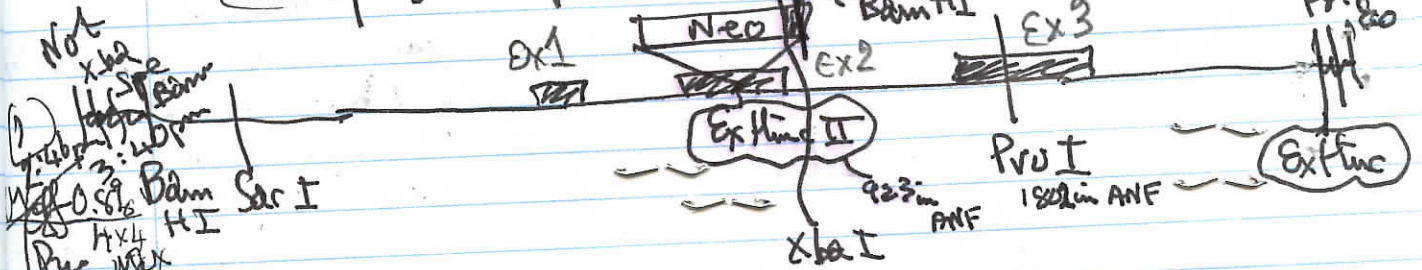
Dye
w
ACR
Not
Sac I
Sac+Nu
Aat II
Aat+Nu
Pvu I
Pvu I+Nu
AG
w
Dye

N2

Tues. Feb. 26th

Not I

Mapping PNTANf3 M'140



ITK

57am Not
11:00 am
0.816 H₂O

Dye

W

ACR

Not

Sac I

Sac+Not

Aat II

Aat+Not

Pvu I

Pvu I+Not

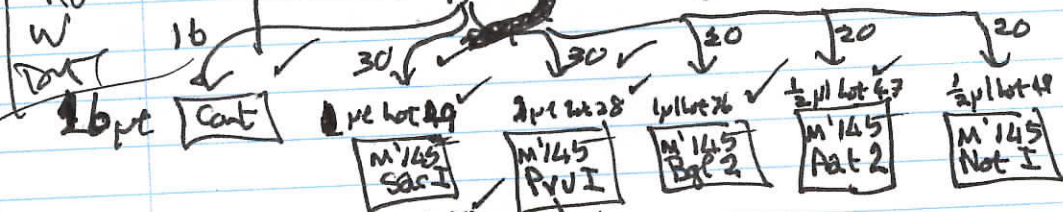
AG

W

Dye

Test Sac I, Pvu I, Bgl 2, Aat 2, Not I
 ↑ ↑ ↑ ↑ ↑
 RI RI RI RI RI

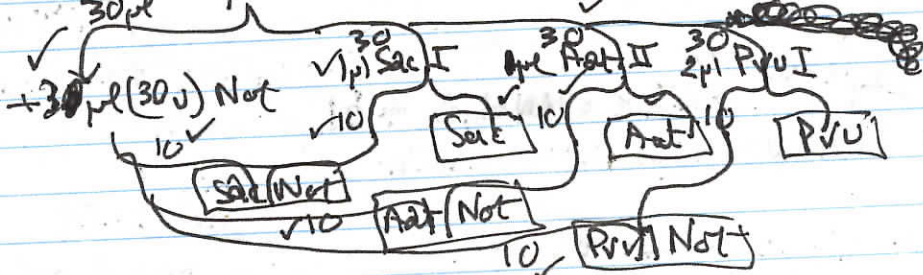
Take 7 µl M'140 ANF 3
 14.1 µl 10x RI salts
 120 µl ddw

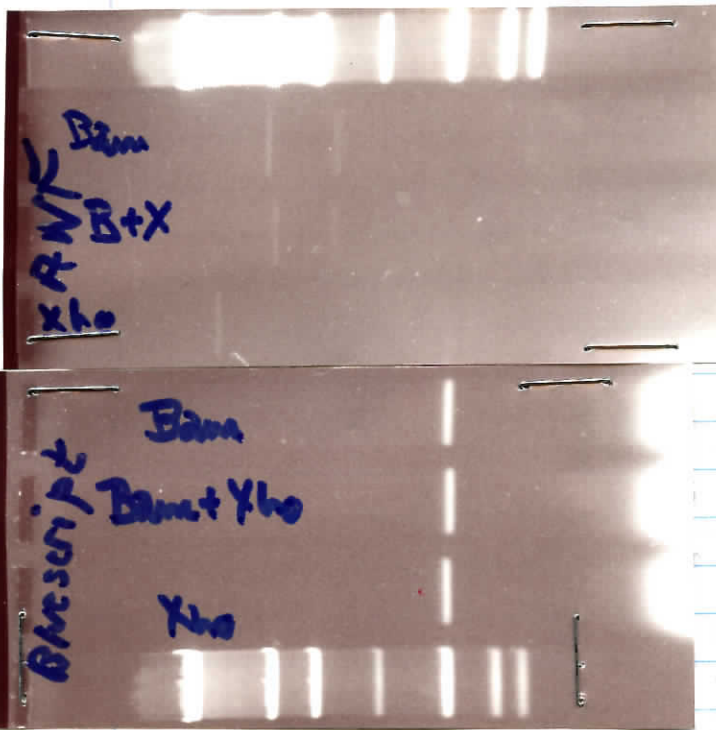


Simon has found & will send the original 10kb Bam fragment

Take 7 µl M'140 ANF 3
 14 µl 10x RI salts
 120 µl ddw

37° 5:22pm





① Conclusion. Bluescript cuts are good. Simon's plasmid is very low conc, but Bam cuts twice, as expected, and Xho cuts once & shortens the 10 kb frag^t. Proceed.

ON 12:56P
OFF 1:34P
0.810 HX4
0.810 M2X

②

Dye
W

ACR

W

M'147 B+X

M'146 ANFBX

W

AG

W

Dye

② Conclusion:

The BXC & EtOH pptⁿ worked well. Proceed.

2+12

Howe's Medical Institute
Research Laboratories
Simon W. M. John, Ph.D.
Assistant Investigator

Dear Oliver,

Attached is the ANP BamHI fragment in a CseI purified plasmid. I do not have it in bacteria. The plasmid is one of the ori varieties. The person who gave me the ori did not know the variety. My digests suggested it is ori EH100. The plasmid also contains an approximately 6kb BamHI fragment from somewhere in the genome. This fragment was somehow seems to allow the ANP containing plasmid to grow well. This fragment was not characterized or restriction mapped (I purified the ANP fragment for mapping).

I did not calculate the concentration of DNA in this prep but 3 microlitres was bright on a gel. I do not have any more of this prep but have a small amount of a different prep.

Good luck and best regards

Simon

Simon

FRam ✓
HI ✓
at back into solⁿ at 5:50 10:10am to 10:50
1x φOH/HOQ, 2x CIAA ✓
IN H₄Ac + 1 pl EtOH. Freeze - Cent. Dry

Back into 10 pl 0.001M EDTA as

M'146
ANFBXC

M'149

✓ Run gel on 2 pl vs. M'147 BS B+X

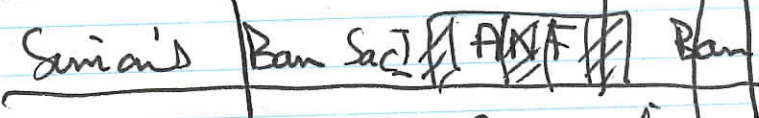
Grow up more of Simon's plasmid

(1) 3:42 pm
 4:55 pm
 0.86 max

Wed. Feb 27th

Xho I 147

Pvu I



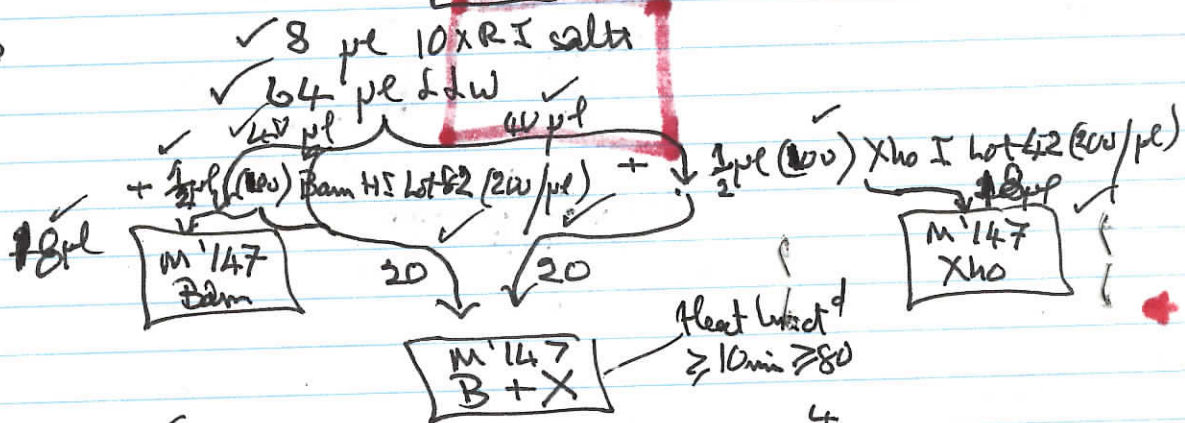
Byg
 w
 AGMM
 Bam
 Bam + Xho
 Xho
 Bam
 Bam + Xho
 Xho
 AG
 W
 Byg

When the arriver need to check for possible
 Sac I to Pvu I 3' limit of ~ 5.8 kb & Bam to Sac
 5' channel (shortened at Bam end for primer) of
 about 800 bp. Test also whether the
 Sac - Pvu has any Mlu, Bsp, Xho or Hind III sites for
 pos. use to insert Amitoc, AN fragment from
 M92 for M23.

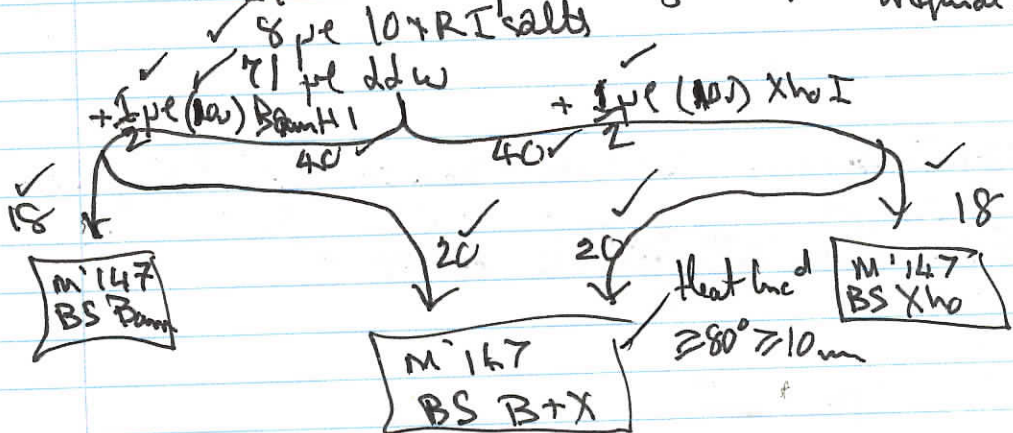
Bluescript Try Bam, Xho & Bam + Xho on Serian's clone
 and if okay purify Bam Xho for subcloning in Bluescript;
 it may work without ~~purification~~ purification if vector is CIAPed.

M'147
 ANF
 Bam
 is in an
 overvector

Take 8 µl M'147 ANFB8 3' limit angel 1/5/92
 ANF
 Bam ANFB8 (scl) / did 2-23



Take 1 µl Bluescript M'147 from Seige BS-II-KS(+) K-64 original.



3:70 1:10 pm
 3:20 pm

(1) ~~04/12/38~~
~~08/11/23~~
~~08/08/90~~
 By
 W
 M'149 A
 M'149 A
 W
 AG
 W
 date By J
~~2:27 PM~~
~~08/08/90~~
 s
 By
 W
 ACB
 rect.
 ced.
 By
 W
 ACB
 Ban
 Rh
 M'149 BX
 m
 26
 W
 W
 By

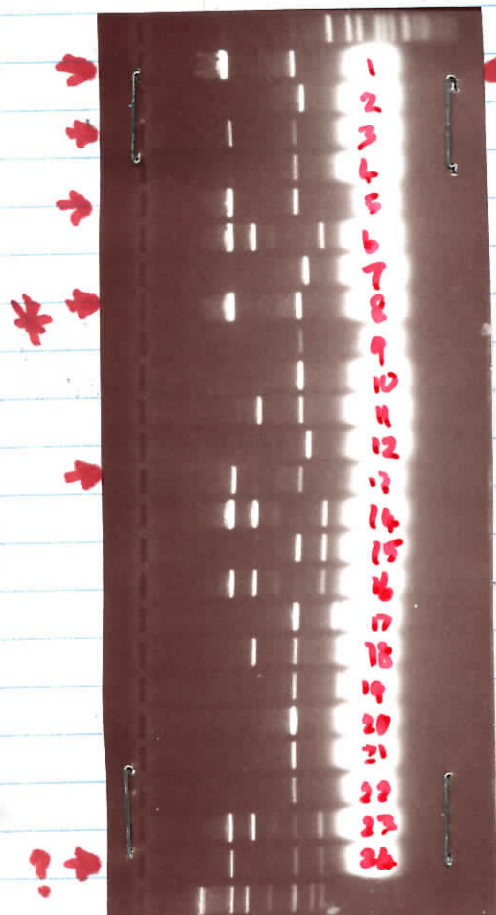
Cont
Bx?

$x/4$
Max

$$\left(\frac{5}{16}\right)u$$

21
is v
2b
ok
37
M 14

To
int
w



② Conclusion

② ⑦ ⑩ ⑬ are probly in vector

④ ⑨ ⑩ ⑬ ②① ②② are the script or date

⑥ ⑭ ⑮ ②③ are Simon's original

⑤ ⑧ ⑨ contain different small frag^{ts} in ps

⑪ ⑮ have a medium frag^c

* ① ③ ⑤ ⑦ ⑬ & (unpure) ②④ are correct sequence

⑧ (Pool 1) ③ ⑤ & ⑬ proceed

M'149 Pool
ANFBXC

M'149
(8)

Baum Xho
fragt

Diagram illustrating the components of a system:

Inputs: B and X

System: SC ANF

Output: Y

Block: BS

Take 50 μ l 10X RT buffer + 20 μ l 2.5 μ l (50u) Bam HI 2.5 μ l (50u) Xho I
1 μ l M149 ANFBXC minus 37° 11:50 to 2:05p

(4) (9) (10) (20) (21)
 ↑ Blue
 (4) (9) (10) (20) (21)
 ↑ Mixed blue & white

3/9
2/8

Hx4
Max 3/10 RNP set

Thurs. Feb 28th

A/Cant
A/B+X

M'147 ANF Bam scraped

Transform

M'147 ANF Bam

Sam's original in Bam or vector

2/26 M'149 DH5 α
M'147 ANF Bam

80 μ l ✓
2 μ l ✓

37° 10:05^{am}

0° 9:31^{am} to 9:57^{am}
42° 90 sec.

R, 20, 5, 20, 5/16

Many

(21) Not expected

8/9/10 Hx4
Max

Plated (with extra 1X NZY) on 2X AMP NZY

(5/16) picked for regular growth. RT 9:30^{am}. Grow up if mini is not good.
5, 20, 5/16 plates scraped (+satellites) & prepⁿ made $\approx$ 3 minis (N.M. protocol).

Bam + Xho
BXC mini

M'146 ANF BXC ligation etc.

M'149 ANF BXC

Red label

Take 9 μ l (whole) of M'146 ANF BXC (Cipped)

M'149
ligation
ANF BX

+ 3 μ l M'147 BS B+X (heat inact)

+ 1.5 μ l 10x P95 ligation buffer

+ 1.5 μ l M'77 T4 ligase

15° 2:58^{pm} RT 4:06^{pm} 37° 4:43^{pm}
Truncated ligation 1, 1/2, 1/4.

Transform

M'14

10/10 to 20
is white. Picked
24 with some difficulty
at 1^{pm}
37° at 2^{pm}
M'149 ANF BXC mini

M'149 DH5 α

80 μ l ✓

50 μ l standard DMSO
Xgal / plate

ANF BX M'149 ligation

70 μ l ✓

2X AMP 1X NZY 37° 5:45^{pm}

0° 5:14^{pm} to 5:35^{pm}

90 sec 42°

R, 20, 5, 20, 5/16

Fri. March 1st

Take 10 μ l M'147/9 ANF Bam

+ 20 μ l 10x RI salts

+ 170 μ l dH₂O

+ 9 μ l Bam

HIT Lot 82 (20 μ l)

+ 2 μ l XhoI

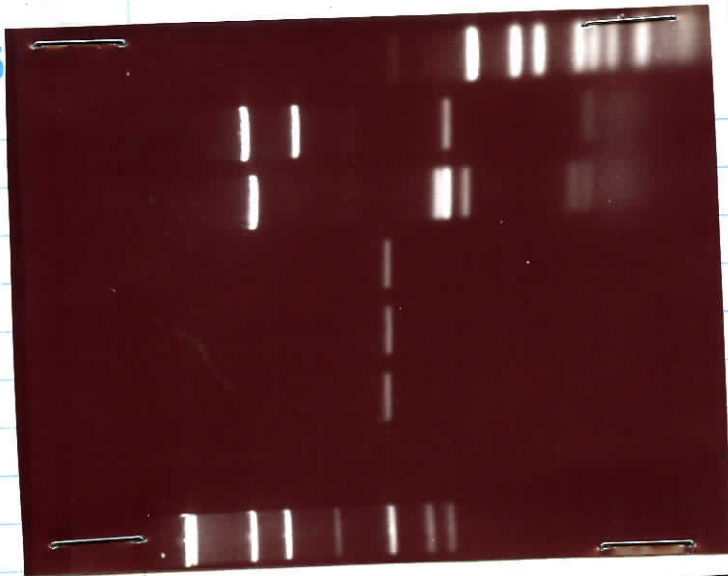
Lot 42 (20 μ l)

To sequence into Bam site
w/ F1 primer
Forward

M'149
A/Cant

M'149
A/B+X

37° 10:33^{am}
Frozen 11:38^{am}



① Conclusion: Sac I site is there - likely ~ 480bp from Bam HI
Bam + Sac on BSS OK.

M13 F1 primer goes
Sac I Not I Xba I Bam HI

236. seq	ANFBX			Water 14 µl ✓
	M'149 (8) ✓ 1 µl	M13 F1 primer (M'150) ✓ 5 µl		

237. seq				Water 14 µl ✓
	M'149 (8) ✓ 1 µl	M13 R3 primer (M'150) ✓ 5 µl		

240. seq	See M'154	No sequence
-------------	----------------------	-------------

M13 R3 primer goes
Kpn I Xho I Apa I

Submitted March 5th

Stopped at 1st of 6's

Submitted March 7th

32184

	M'150	Blue ANF470
1st	G C A	G G C A G A T T T C T G
2nd	A G T	T C A
489		

Bam HI

04:10:30am
 off 11:50am
 0.86g X 4
 3/10 RNAse
 max

Fri - March 1st

①

Bam

ACR

M'151 pte Sac

M'151 A/B+X+Sac I

M'151 BS Bam HI

M'151 BS B+S Take

M'151 Sac I

w

AG

w

Dye

Bam $\rightarrow$ $\leftarrow$ Xho

Sac I

check of this.

10 μ l M'149 A/B+X (gel as M'148)+ 4 μ l ~~8 M NH₄ Ac~~~~8 M NH₄ Ac~~ + 110 μ l EtOH

Freeze - Cent - Dry

Back into

40 μ l 1x NEB buffer 1 + 1x Alb32 μ l H₂O 4 μ l 10x NEB 4 μ l 10x Alb(10 μ l) Sac I Lot 49 (20U/ μ l)

Blue label

M'151
A/B+X+SM'151
A/B+X+S

37° 4:08pm to 5:11pm Frozen

In preparation for subcloning Bam — Sac, if successful:—

Take X μ l Bluescript M'147X μ l 10x NEB buffer 1X μ l 10x Alb.X 63 μ l ddw

Red label

40X

40

1/2 μ l (10U)
Bam HI
Lot 82 20U/ μ lM'151
BS Bam

20

M'151
BS B+S

20

Red

1/2 μ l (10U)
Sac I
Lot 49 20U/ μ lM'151
BS Sac I

37° 4:18pm

5:25pm
FrozenHeat inact! $\geq 80^\circ \geq 10$ minSunday
March 3rd

Repeat "Red label" with Xho I & Sac

Xho Lot 42 (20U/ μ l)

Blue

Blue

M'151
BS XhoM'151
BS X+SM'151
BS Sac I1/2 μ l Sac I

37° 11:08am

12:22pm

Sat - March 2nd

Same thoughts.

Need to be sure that Bhr GN
really parallels BMHC and that A (into) C
parallels AMHC. Says will test by setting
this Tag man.

? whether Bhr GN. A (into) C
and NG 4 G. A (into) C

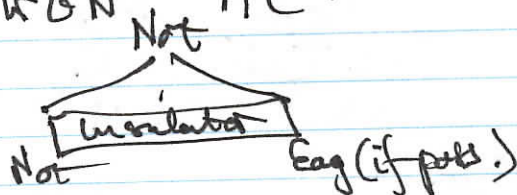
give the same pattern. Tomoko can
turn Bhr GN around with: -

(i) | Bhr GN | AC |
step 1 → ~~Not~~ ← Already made

(ii) Excise Not Bhr GN Not → purify

(iii) Reclone into Not Not AC Not C (AP-ed)
using M'137 protocol.

With Dominic, it would also be good to
test — Bhr GN Not AC —

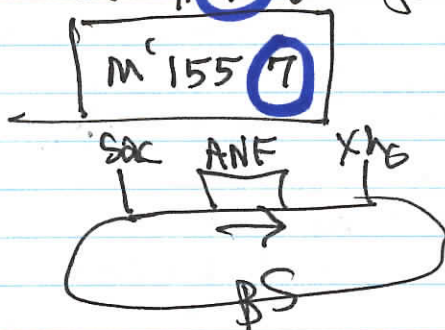


Conclusion:

This was a poor left digest.
 Sh'd have used B+S+X

None of red are likely to be correct - they are too large -
 sh'd be 3 kb (BS) + small B+S.

Nearly all the blue look ok -
 sh'd be 3 kb (BS) + large Sac - Xho
 #3 & #10 may not be the same as each other.
 Take #7 for sequencing



Others pooled

M'155
 SX pool

at string of 6

Submitted
 March 5

238. seq	M'155 ✓ ⑦ 1 pl	M'150 ✓ F1 primer 5 pl	Water 14 pl ✓
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239. seq	M'155 ✓ ⑦ 1 pl	M'150 ✓ R3 primer 5 pl	Water 14 pl ✓
-------------	----------------------	------------------------------	------------------

Submitted
 March 7

240. seq	M'155 ✓ ⑦ 1 pl	M'150 ✓ blue ANF 4-70 5 pl 10	Water 14 pl	seq pool
-------------	----------------------	--	----------------	----------

Sun. March 3rd

Subcloning ^{ANP} Bam - Sac small frag⁺!

Need to heat inactivate M'151 A/B+X+S
 - but better w'd be to start with #8, cut in
 buffer 1 with Bam+Sac+Xho & heat inactivate.
 ligation with blue script ^{Bam Sac} sh'd
 work fine if BS is kept low. ^{BS} + B-S + X-S ^{large frag}

Take 4 μ l M'149 (8)✓ 8 μ l 10x NEB buffer⁺✓ 8 μ l 10x Alb✓ 57 μ l ddw✓ 1 μ l (20v) Bam HI lot 82 (20v/ μ l)✓ 1 μ l (20v) Xho I lot 42 (20v/ μ l)✓ 1 μ l (20v) Sac I lot 49 (20v/ μ l)37° 10:47 to 11:57 Heat inact. ≥ 10 min $\geq 80^\circ$ ✓Take 10 μ l M'155 (8) B+X+S✓ 10 μ l M'151 BS B+S✓ 2.5 μ l 10x p95 lig⁺ buffer✓ 2.5 μ l M'77 T4 ligase

RT to 5:03 37° to 5:24 to cold

Transformation

M'149 DH5d

M'155 lig⁺ ns

05:25 to 6pm

90 sec 42°

B+S

80 μ l4 μ l

6pm

X+S

80

4 μ l

6pm

2x Amp 1x NZY

50 μ l Xgal / plate

beforehand

R, 20, 5, 20, 5

16, 16

37° 6:18pm

each spread immediately. Sun

plus 50 μ l NZY

Both have many white!

Very few blue!

1.3

Bam HI lot 82 (20v/ μ l)B S
+ B-S linker

2:14 PM Will be fine

10x

AG

Blue Xho + Sac

12

13

Red

24

R Bam + Sac

W

32° v/pm

M'155 mini

1 - 12 Blue M'155 X+S

3 - 24 Red M'155 B+S

37° 1:35 pm Mon.

11 1.3 μ l Xho I 198 μ l

Lot 42

20v/ μ l1.3 μ l (25v) Sac I lot 49 (20v/ μ l)Bam HI lot 82 (20v/ μ l)

Wed. March 6th

Review of BS subcloning m155

37° 9:41 am
1
6:52 am

Take ✓ 50 µl 10x NEB buffer 1
✓ 50 µl 10x ALB
✓ 343 µl H₂O
✓ 2.5 µl (50u) Bam HI hot 82 (20u/µl)
✓ 2.5 µl (50u) Sac I hot 49 (20u/µl)
2.5 µl (50u) Xho I hot 42 (20u/µl)

20 µl + 1 µl m155 Blue minus 1-12 → 2 µl Red minus 13-24

R
O
m¹ 155 (8) B+S-X

M' 155 ⑦ 1 pl	M' 159 Blue 10 5 pl	Water 14 pl	} Nile exterior
M' 155 ✓ ⑦ 1 pl	M' 159 Blue 5 10 5 pl	✓ Water 14 pl	

completely
the
sequence.

Sunday March 10th

Completed - 159
March 18th

More sequencing attempts

Review of 236, 238 & 239 sequenced with repeat exclude program from genome center at Washington Univ. shows a really large fraction of 236 & 238 as repeated elements. 239 is unique. Two possible primers in 238 seq are: —

OK

GAA TTA TTT ATT CTA GCT AGC TCA T

M'159 Red 238

as red primer from 200 (red →) 176 of 238 seq.

best good is a blue primer from late in 238; can get something that might work altho it includes some repetitive sequence on its 5' end.

Noting OK

TG CGC CAC AGA CCA GCA A A

M'159 Blue 238

TG CGC C AC AAG ACC AGC A A A

M'159 Blue 238A

An attempt to continue in Red direction on M'155 (7) & in Blue direction after ANF

		M'159 Red 239	
19	63%	CTG	ACT GGG GGT TGG
		GAG	T
		Red End of 239	
		M'159 Blue 3	
20	50%	CTC	AAA ATC GTC TGC
		CTC	CA
		Blue end of mouse ANF	

		M'159 Blue 5	
21	48%	CAC	AAT TAA GCA GTG
		AGC	GCA
		M'159 Red 238	
25	28%	GAA	TTA TTT ATT CTA
		GCT	AGC TCA T
		M'159 Blue 238	
19	58%	TGC	GCC ACA GAC CAG
		CAA	A
		M'159 Blue 238A	
20	55%	TGC	GCC ACA AGA CCA
		GCA	AA
		M'159 Blue 4	
19		GCC	AGC AAG TTC CAG
		AGG	G
		MORE EXT	

Oligo # _____
Date Rec'd _____

LEN: 6 bp
% GC: 50%

Oligo Name: M'160 ~~AAT~~ M'161.

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

Oligo # _____
Date Rec'd _____

LEN: 6 bp
% GC: 38%

Oligo Name: M'160 A f e II N h e I A f e II

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

Thurs. March 16th

anfseqs5

161

Overall view of Bam^{Site} ANF1 Xho Bam

Recent sequencing, combined with mouse genome clone AL606929 allows an overview when checked with Wash. Univ. Repeat Masker program. Upstream of ANF is highly ^{and contains silencers} repetitive. Downstream is unique & runs quite quickly into a chloride channel gene CHCN6 that ends just inside the 3' Bam. Need to check on exact intergenic space & whether CHCN6 is expressed in heart. Promising construct is likely to be

using genomic PCR to obtain the extension beyond 3' Bam.

Non-repetitive

Fri. March 15th

Promising route: (to be checked)

Grown in Dam-

Step 1: excise from anfseqs6 ANF1 3kb CLAI 5210 BspBI 8100 and clone into Cla site of bluescript.

Step 2: change BspBI to MluI and excise for use as control short arm MluI 6490 ARII ARII NheI 7840 (B)

Step 3: change AgeII (sites) to NheI and excise for use as experimental short arm MluI 6490 AgeII NheI 7490 (A)

Step 4: single cut AscI DupDel with NeopeA in red direction and complete cut NheI (Buffer 4) (A) (B) clone into AscI NheI sites the two short arms. Can use 4B as control

Step 5: Bam ANF1 BspBI 5550 SspI 6420 change to Xho & excise Bam... Xho or Bam... Xho SspI

Step 6: Excise from M92 Xho Antof AN Mlu

Step 7: Cut Step (4A) ~~with~~ with Bam and AscI and assemble 3 pieces NotI Bam Bam ANF1 Xho Xho Antof AN Mlu AscI Neo PolA AscI short arm NheI