

Smith

April 5th 2001

Dept. of Pathology

UNC - Chapel Hill

Chapel Hill

NC27599-7525

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? glycerol in Page I
- but also Part II

① Conclusion: There's
same electrophoretic
anomaly going on.
Streaked out #4
as MR_{CTA} & #18
as YN_{CTA}

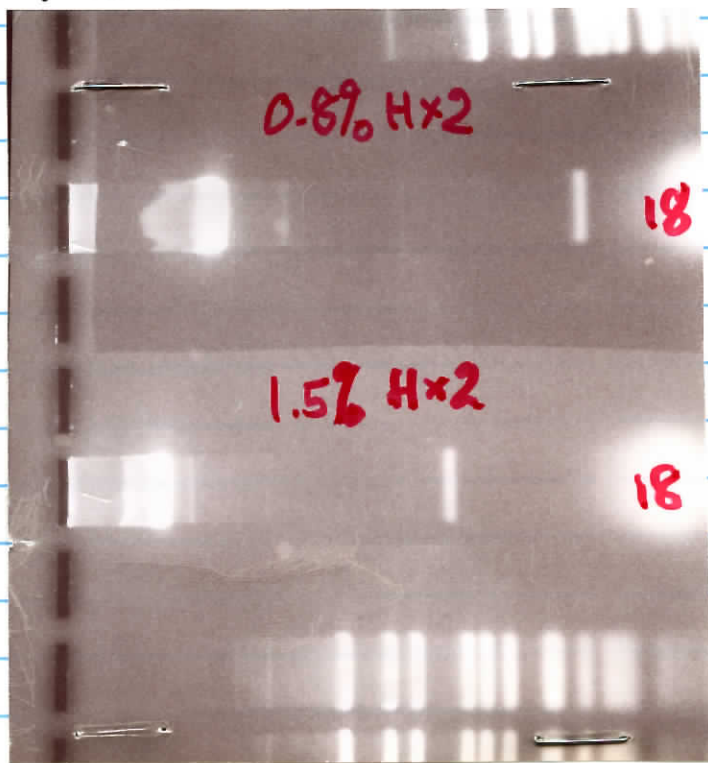
Recheck with 0.8% Hx4
To Seig as single colonies

L'1 #4
MR_{CTA}

L'1 #18
YN_{CTA}

Previous K'161 MR_{CTA} #2 was
almost certainly stay also.

Gel (b) from L'3



On 7:34 PM
 887:54 PM
 1503 (ORNAse max)

Thurs. April 5th

Retransformations for **MR CTA** & **YN CTA**

Age	Strain	Notes	MR CTA	YN CTA	Notes
(1)	MR CTA	Domestic says they're v. good.	80 pl	80 pl	1 tube XL-10 Gold 10 min
(2)	MR CTA	L1 DH5α	5 pl	5 pl	80 pl
(3)	YN CTA	K'161 lig ⁺	—	—	80 pl
(4)	YN CTA	K'159 lig ⁺	—	—	3 pl
(5)	MR CTA	0° 3:27 pm to 4:15 pm	—	—	12:45 pm
(6)	YN CTA	42° 90 secs	—	—	1:15 pm 30 secs 42°
(7)	MR CTA	Plated 1x Amp NZY	—	—	4:30 pm
(8)	YN CTA	—	—	—	4:30 pm

Repeat with "Gold" & setup new ligations if unsuccessful.

Repeat YN CTA R, 20, 5, 20/16, 5/16 L1² 2x Amp NZY

Estimate 100x better

MR CTA R, 20, 5, 20/16, 5/16 L1² 2x Amp NZY

Sat

Picked L1 (1) - (12) MR CTA min 37° 6 pm

L1 (13) - (24) Blue min 1x Amp NZY

Age/Bsi Mix	Take	Notes	Age/Bsi Mix	Take	Notes
26 pl 10x RI	✓ 21 pl 10x RI	(20 pl)	26 pl 10x RI	✓ 21 pl 10x RI	(20 pl)
15 pl (30v) Age	✓ 15 pl (30v) Age	Hot 33	15 pl (30v) Age	✓ 15 pl (30v) Age	Hot 33
30 pl (30v) Bsi VI Lot 18	✓ 30 pl (30v) Bsi VI Lot 18	(100 pl)	30 pl (30v) Bsi VI Lot 18	✓ 30 pl (30v) Bsi VI Lot 18	(100 pl)
YN min 3 pl to 20 pl	YN min 3 pl to 20 pl	37° 30 to 55° 30 to 16 pm	MR min 3 pl to 7 pl	MR min 3 pl to 7 pl	37° 30 to 55° 30 to 16 pm

Conclusion: (4) & (18) are both correct. - 2.00

Boyle
W
ACR
~~W~~
[4
18
W]
Fresh
~~W~~
1.5% Lot
with 6.5
fisher
Low EO
agony
new knife

Degeht

(2)	W	old
{	(4)	1.5%
	(18)	wed
	W	on L1

(2) Conclusion - sample does look to be somewhat related to agarose. Try a different lot.

4

③ m
ACR Nelson
w against
④ w. 6842
w.
Dye

Error
 2 1/2 min.
 at 10V
 but don't
 3
 Repeat
 will be
 1 to 10V

③ ~~Looks more like an entry problem.~~
~~One more test. Start at $1.6V$ an~~
 ~~$\uparrow$ to $1.6V$ an after 5 min.~~

④ ← Did it help, did test
was imperfect. → Certainly
the problem is related to entry,
Tried 20 min at 13,000 rpm.
40

40

cross-section:

Mon. April 9th

MP_{CTA}

YN_{CTA} (cont.)

(Using less enzyme & more mini)

On 2:30-3:30 Hx4
0.86 Hx4

Age ①

W AG

Pre ④

Post Aat ④

Post Aat / Bsi ④

Post Age / Bsi

W L¹³ YN ⑧

ACR W

Age

18pe

L¹³ MR

Pre

3/10 RNAse

Back into 100 µl 1x R1 salts

+ 2 µl (20u) Bsi W Aat

Lot 18 (10 u/µl)

Take 10 µl 10x R1 salts

✓ 63 µl ddw

✓ 15 µl L¹³ YN_{CTA} #18

✓ 10 µl Age I Lot 33 (20 µl)

✓ 2 µl Bsi W Lot 18 (10 u/µl)

37° 9:35 to 11:50 am

55° 12:45 to 2:15 pm

55° 12:05 to 1:40 pm

Well into gel

④ Cont.

⑧ Uncont.

W ACR

Bye

Wed April 11

On 11:30-12:11

0.86 Hx2

1.5% Hx2

7u/cm

ACR L¹³ MM



⑤ still no effect. Try Hx2.

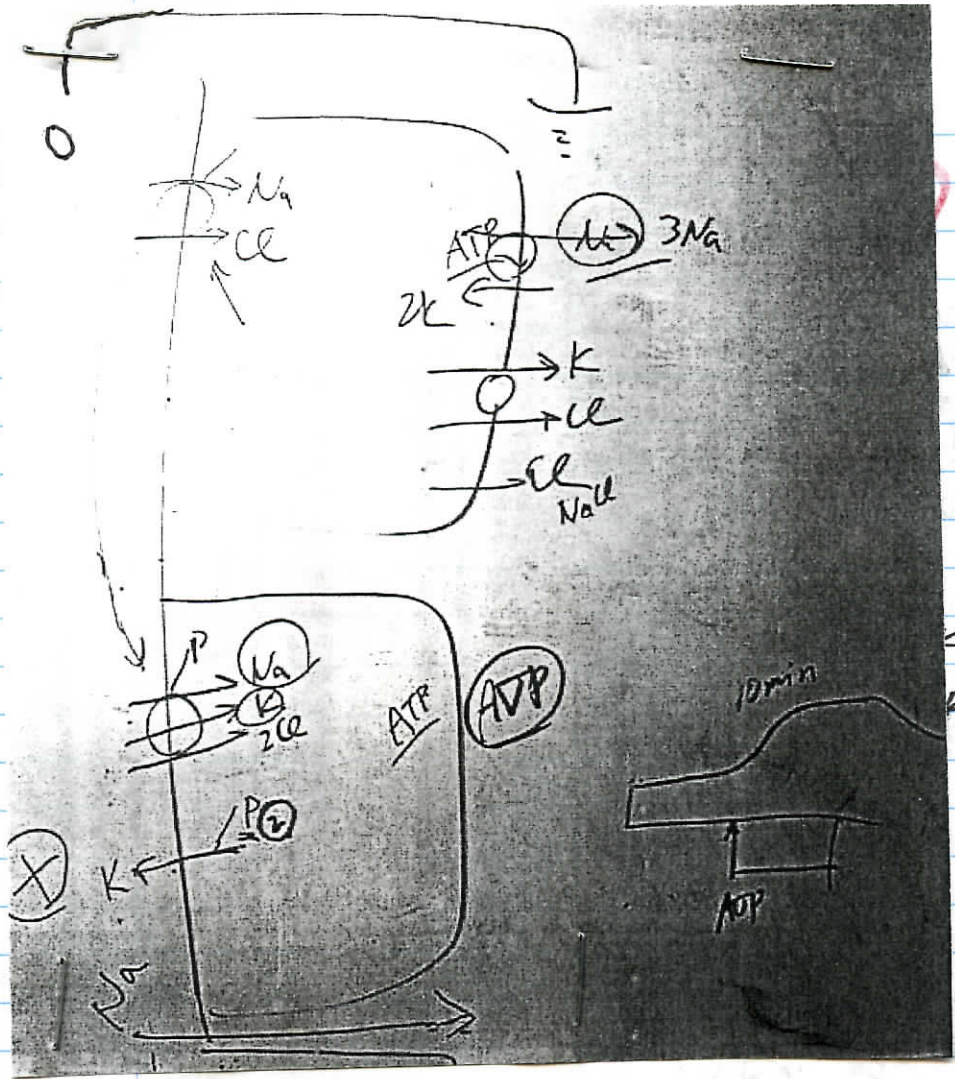
Full photo on L¹³. The buffer fixed the problem!

L¹³ MR Aat ④ Bsi

18pe L¹³ MR Post-Aat

L¹³ YN ⑧ CTA Age Bsi

4



10 min -
2 to 3 hrs.
Tubular Na^+ in response to AVP

lower cell pumps $2 \times \text{Na}^+$ for same ATP because of electrostatic gradient.

Tues. April 10th

NKCC2 models (contd.)

Arly Anderson visited & we discussed. Afterwards Nishiyuki drew the left page diagram & I stopped him & suggested an equil⁻ between two cell states - because of ab studies with ROMK showing two types of cell. Hypothesis is that NKCC2 +/- changes proportion of lower cell type & so pumped more Na^+ than will be expected. ? AVP determined. Testable!

Another case of possible switch between two states for a cell with the intracellular signal being PKA or whatever.

All cells express NKCC2

Ab against NKCC2

Ab against ROMK

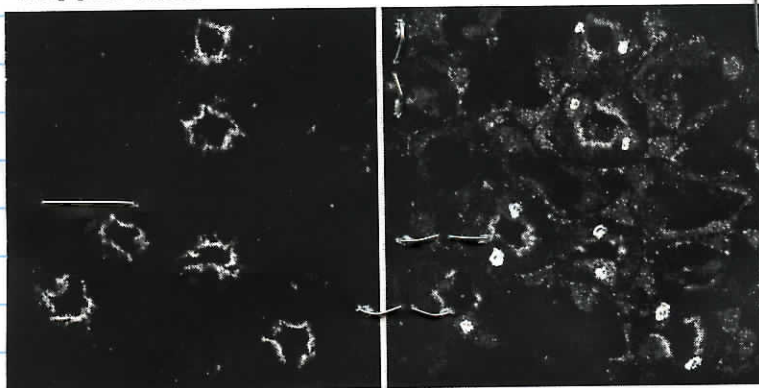
Some cells do not express ROMK

Nok15g g=L320 NKCC2

#5 (+/+)

Nok15r r=Lc35 ROMK

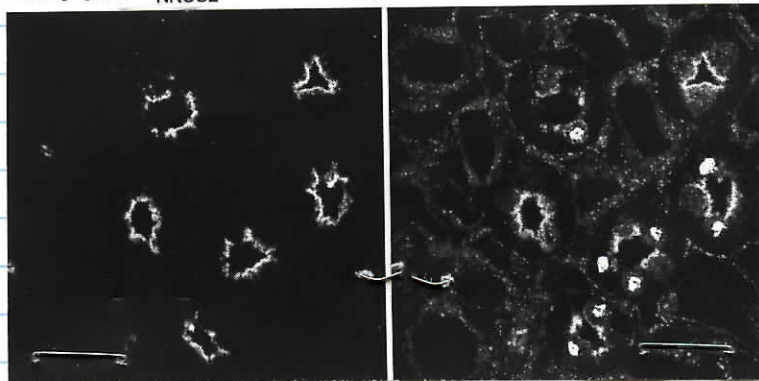
d41



NKCC2
+ / +

Nok17g g=L320 NKCC2

#2 (+/-) Nok17r r=Lc35 ROMK



NKCC2
+ / -

[illegible]

L'7 CTBlue & K17RedM10
 Generic
 for any
 CODETEST constructs
 $\neq$ pde A.

[illegible]

BSI WI was put into Poam site
→ loses STOP codon. Shd
have used BDI.

1324
• ECFP → TAC AAG TCC GGA CTC AGA TCT CGA GCT GAT CCA AAA AAG AAG AGA NLS
BspE I Bgl II Xho I

1369
• → NLS → NLS
AAG GTA GAT CCA AAA AAG AAG AGA AAG GTA GAT CCA AAA AAG AAG

1414
STOP
AGA AAG GTA GGA TCC ACC GGA TCTAGATAACTGATCA
BamH I Xba I* Bcl I*

159.89 (Revered)
CN CTA
Seiga

[illegible]

BScWI

SV40 NLS
in yellow

7 Red Mlu

Thurs. April 12th

Check on CN_{CTA}

Can use with all
CODETEST for
TESTCODE

Because of current lack of fluorescence,
Seigo will sequence current CN_{CTA} with K'17 Red Mlu
primer. Meanwhile make L'7 CT Blue primer
for use with all CODETEST (not TESTCODE) constructs.

This is over Bacter
splice acceptor region.

Steps 28645 4/12/00

length 20

Oligo Name: L'7 CT Blue 50% GC

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

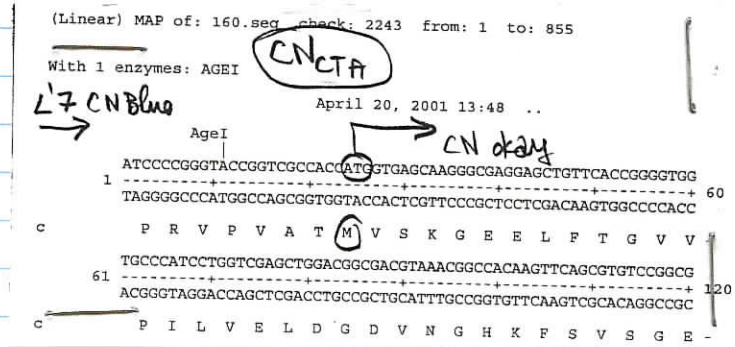
Wed. April 18th

The sequencing with K'17 Red Mlu showed the
error. I changed Bam to Bsi instead of
BqI to Bsi & so wiped out the stop codon.
Return to the top & repeat!

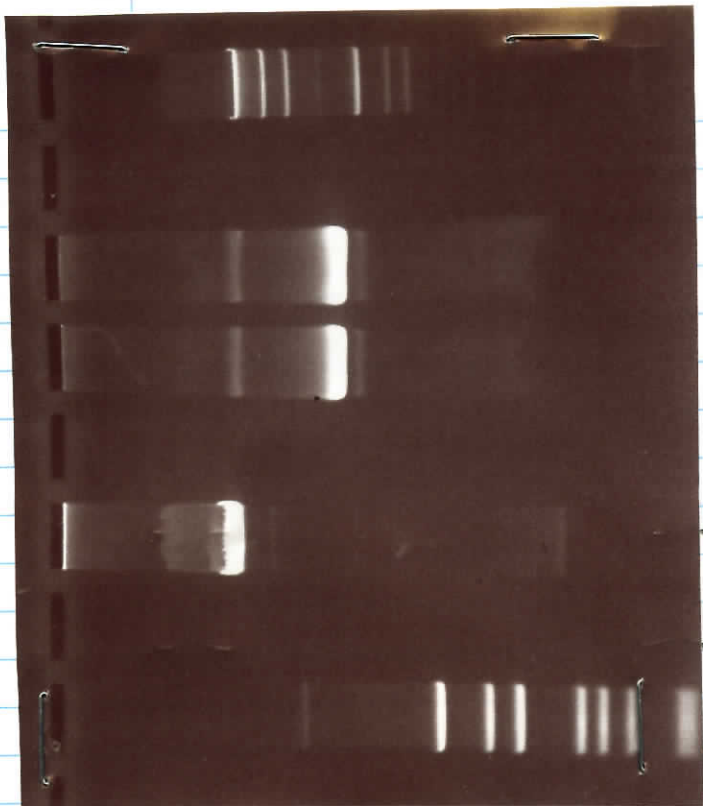
L'7 CT Blue works fine!

Comment Sequencing is insensitive to
primer conc. Accidentally
used w/100% for 159. seq & it worked.

160. seq (Forward)
CN_{CTA}
Seigo.



8



① Conclusion: Try again with
new lot of Bcl I. Gel
didn't help.

Tamoko's notes
check that FB 446
I'9 was used.

①
10/18/87

CN

Saturday, April 21st

CN again

BsiWI

↑

BclI

CN

46

Take 6 pl NEB Buffer 10X

48 pl ddw

6 pl

NucP ECFP Dam⁻ T#3-89 (faded mini)

1.5 pl (150) Bcl I Lot-16 (100 pl) (1995)

Because of age

55°C 19 am to 11 am

18 pl

① 11:10 am
11:30 am
1.5% H₂O
GIBCO Agarose from S. Lord

L9 Pre

100 pl ACR 3/10

L9 Post

Dye

W

Agarose

L9 Pre

Bcl I of NucP ECFP

L9 Post

W

① from L3 - std. for gel test to test this

GIBCO Agarose ex. S. Lord

W

ACR MM

W

Dye

Wed - April 25th

Checking back shows that Sergio did not
use J9 & got a lot of good
colonies versus v. few & poor (i.e. okay!)
on J9. The mini were almost
certainly DH5α.

Sunday May 6th

40

horn Field's use of α -MHC [Neo] selection
to "induce" myocytes in ES cell culture systems:-
"select"

α -MHC Pro [Neo] β -MHC Pro [Neo] will allow

$\rightarrow$ & $\leftarrow$ (as below)

But can also start with ES, Neonatal
or Adult hearts $\rightarrow$ we are inducible or
transient α -MHC Pro MAPK Kinase

or β -MHC Pro MAPK Phosphatase

$\rightarrow$ go $\rightarrow$ & $\leftarrow$

Probably the α -MHC IRES GFP
 $\rightarrow$ β -MHC IRES YFP

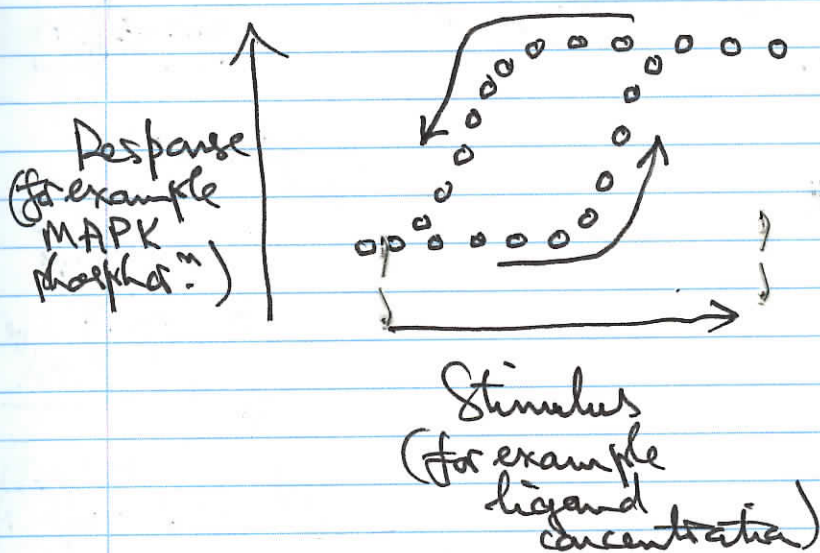
are safer than α -MHC GFP β -MHC
duplication.

α -MHC Pro
 α -MHC
MAPK Kinase
MAPK Phos
+/- KO
Neo
ES or Adult

Wed. April 25th

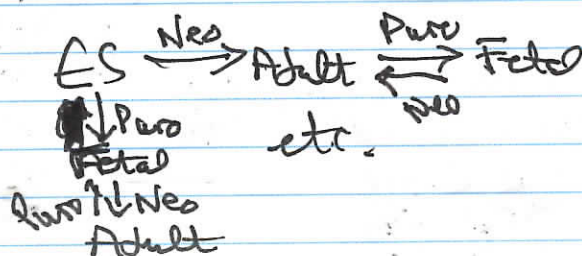
Hypothesis

An essential part of the 2-state hypothesis is hysteresis — the forward and backward paths are different!



Mon. April 30th

A paper by Klug et al. J.C.I 98 (1998) 216-224 shows ES cells to cardiac myocytes by selecting with G418 on α MHC Pro Neo transfected cells. Suggests that when (in reverse) in pSKB1, the following might allow flip-flop



α vs. β MHC
(same level of gene exp.)
same type of function
might be a very good pair of genes.

1951 2 24 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 99 100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180 181 182 183 184 185 186 187 188 189 190 191 192 193 194 195 196 197 198 199 200 201 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216 217 218 219 220 221 222 223 224 225 226 227 228 229 230 231 232 233 234 235 236 237 238 239 240 241 242 243 244 245 246 247 248 249 250 251 252 253 254 255 256 257 258 259 260 261 262 263 264 265 266 267 268 269 270 271 272 273 274 275 276 277 278 279 280 281 282 283 284 285 286 287 288 289 290 291 292 293 294 295 296 297 298 299 300 301 302 303 304 305 306 307 308 309 310 311 312 313 314 315 316 317 318 319 320 321 322 323 324 325 326 327 328 329 330 331 332 333 334 335 336 337 338 339 340 341 342 343 344 345 346 347 348 349 350 351 352 353 354 355 356 357 358 359 360 361 362 363 364 365 366 367 368 369 370 371 372 373 374 375 376 377 378 379 380 381 382 383 384 385 386 387 388 389 390 391 392 393 394 395 396 397 398 399 400 401 402 403 404 405 406 407 408 409 410 411 412 413 414 415 416 417 418 419 420 421 422 423 424 425 426 427 428 429 430 431 432 433 434 435 436 437 438 439 440 441 442 443 444 445 446 447 448 449 450 451 452 453 454 455 456 457 458 459 460 461 462 463 464 465 466 467 468 469 470 471 472 473 474 475 476 477 478 479 480 481 482 483 484 485 486 487 488 489 490 491 492 493 494 495 496 497 498 499 500 501 502 503 504 505 506 507 508 509 510 511 512 513 514 515 516 517 518 519 520 521 522 523 524 525 526 527 528 529 530 531 532 533 534 535 536 537 538 539 540 541 542 543 544 545 546 547 548 549 550 551 552 553 554 555 556 557 558 559 560 561 562 563 564 565 566 567 568 569 570 571 572 573 574 575 576 577 578 579 580 581 582 583 584 585 586 587 588 589 590 591 592 593 594 595 596 597 598 599 600 601 602 603 604 605 606 607 608 609 610 611 612 613 614 615 616 617 618 619 620 621 622 623 624 625 626 627 628 629 630 631 632 633 634 635 636 637 638 639 640 641 642 643 644 645 646 647 648 649 650 651 652 653 654 655 656 657 658 659 660 661 662 663 664 665 666 667 668 669 670 671 672 673 674 675 676 677 678 679 680 681 682 683 684 685 686 687 688 689 690 691 692 693 694 695 696 697 698 699 700 701 702 703 704 705 706 707 708 709 710 711 712 713 714 715 716 717 718 719 720 721 722 723 724 725 726 727 728 729 730 731 732 733 734 735 736 737 738 739 740 741 742 743 744 745 746 747 748 749 750 751 752 753 754 755 756 757 758 759 760 761 762 763 764 765 766 767 768 769 770 771 772 773 774 775 776 777 778 779 780 781 782 783 784 785 786 787 788 789 790 791 792 793 794 795 796 797 798 799 800 801 802 803 804 805 806 807 808 809 810 811 812 813 814 815 816 817 818 819 820 821 822 823 824 825 826 827 828 829 830 831 832 833 834 835 836 837 838 839 840 841 842 843 844 845 846 847 848 849 850 851 852 853 854 855 856 857 858 859 860 861 862 863 864 865 866 867 868 869 870 871 872 873 874 875 876 877 878 879 880 881 882 883 884 885 886 887 888 889 890 891 892 893 894 895 896 897 898 899 900 901 902 903 904 905 906 907 908 909 910 911 912 913 914 915 916 917 918 919 920 921 922 923 924 925 926 927 928 929 930 931 932 933 934 935 936 937 938 939 940 941 942 943 944 945 946 947 948 949 950 951 952 953 954 955 956 957 958 959 960 961 962 963 964 965 966 967 968 969 970 971 972 973 974 975 976 977 978 979 980 981 982 983 984 985 986 987 988 989 990 991 992 993 994 995 996 997 998 999 1000 1001 1002 1003 1004 1005 1006 1007 1008 1009 1010 1011 1012 1013 1014 1015 1016 1017 1018 1019 1020 1021 1022 1023 1024 1025 1026 1027 1028 1029 1030 1031 1032 1033 1034 1035 1036 1037 1038 1039 1040 1041 1042

L'13
CN Pool
BsiWI

← Use #9

L43 (CN) FRAG⁺

③ Good cut

③ On 10:22 am
@ 10:51 am
2V/am

(3)

On / Off

V/am

n08

H x I
eye 1/PK
w

SATY

w

Bye

(Handwritten notes on lined paper)

evaluated
to
50pl
as

L'53
FRAGT
CN

~D.8

Hx1 LIP
eye 7RPM

w

↑
↓

w

Bye

Damaged 23
lost 24

Sat. April 28th
Take

✓/50 ml 10% RI salt

✓ 420 ml hda

✓ 25 pc (50v) Age I lot 33 (2v/pc)

✓ 5pe (50v) Bc/WI Lot B (10v/pl)

20 plabiquets + 2 pl L13 $\text{D} - \text{D}$ minus

32° 12' 50" $\times$ 55° 16'

~~25 pl ✓
 210 ✓
 13 pl ✓
 2.5 pl ✓
 25 pl L134 ✓~~

5pc L13#9

5:40 pm 37°
6:50 55° 2:5

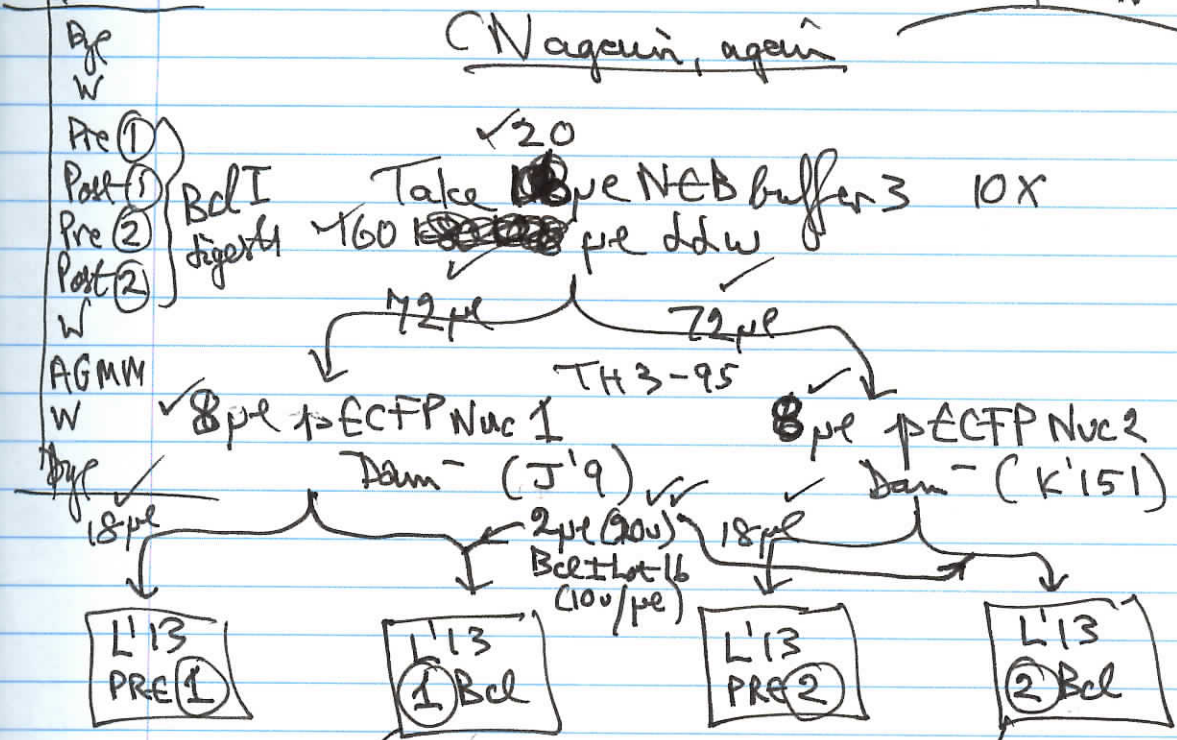
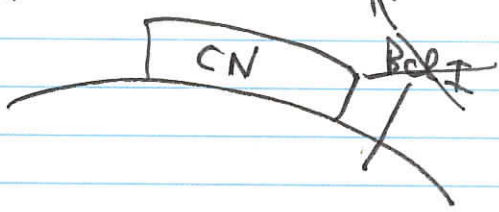
① 2pm
 1.5% H₂O
 100/μl

CN

Thurs. April 26th

Bsi W.I. 13

CN again, again



55° 12:33pm to 1:33pm

KAN

② 4:04
 1.5% H₂O
 3/10 RNAse
 Take 20 μl L'13 ①+② Bcl I CIAA

Age Bsi
 ① Age Bsi
 ② Age Bsi

✓ 4 μl K'117 Bcl I → Bsi W.I. oligo
 ✓ 3 μl 10x p95 buffer
 ✓ 3 μl K'59 T4 ligase
 15° 3:07pm to 4:10pm
 RT 4:10pm to 5pm
 32° 5pm to 5:40pm to cold

L'13 lig.

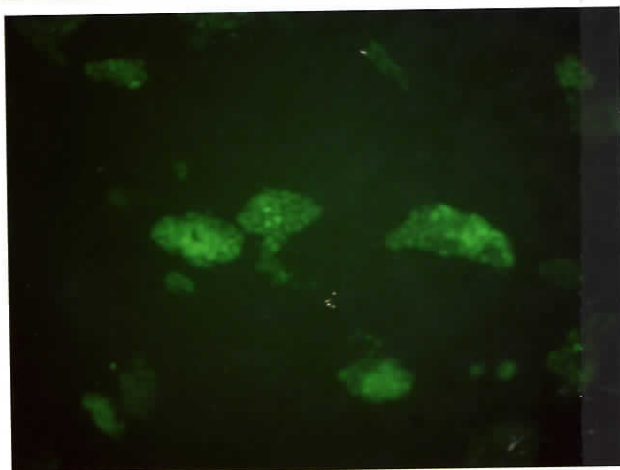
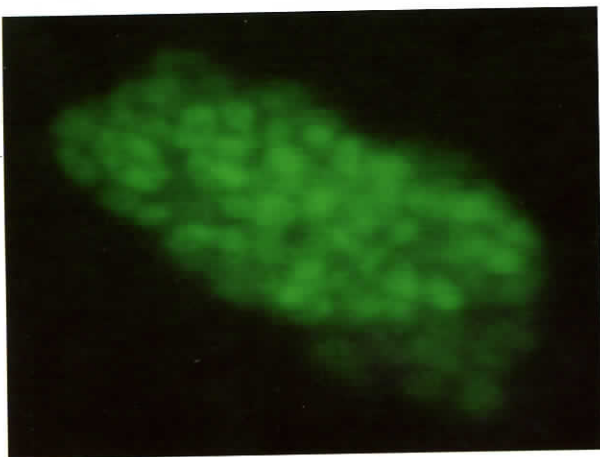
K'53 3H5 d
 L'13 lig.
 80 μl ✓
 4 μl
 0.5 μl to 6:05pm ✓
 90 sec 42°

L'13
 Minut ①-④
 30 μg/ml KAN
 NZY 32° 11:20am

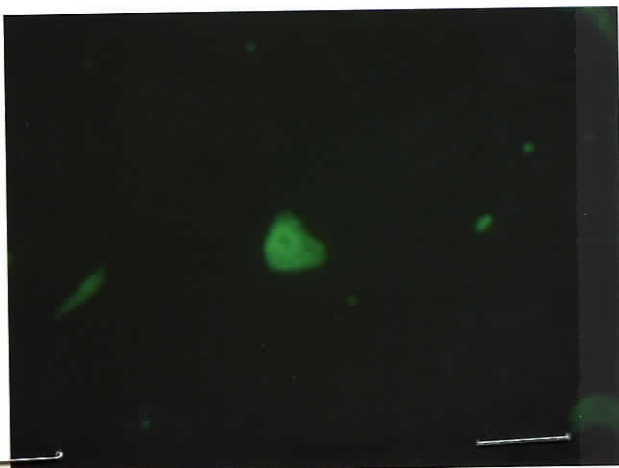
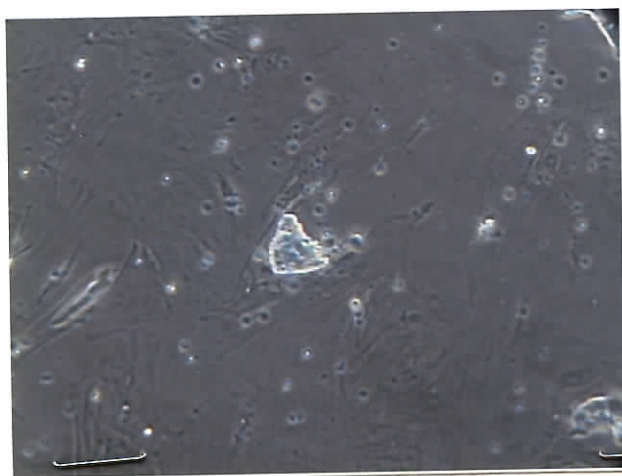
Plated ~ NZY
 KAN
 3/27/01

R, 20, 5 20/16, 5/16 37° 6:15pm
 10:45am

hrGN, small clones



Ycta, small colony



Sat. April 28th

15

(hr GN) reasonably well expressed in nuclei.
Colony number is okay.

(167. seq) hr GNcta (^{sequence is} reversed) sequenced by Seigo
with K'17 Red Mlu primer.

Checks out OK. Rev POLA & GN are
there, but it does have

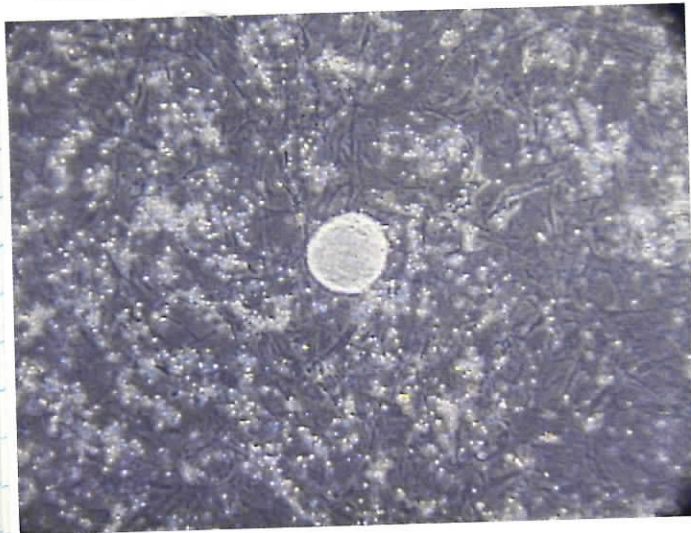
PKKK(S)KV

← Not R, as in SV40

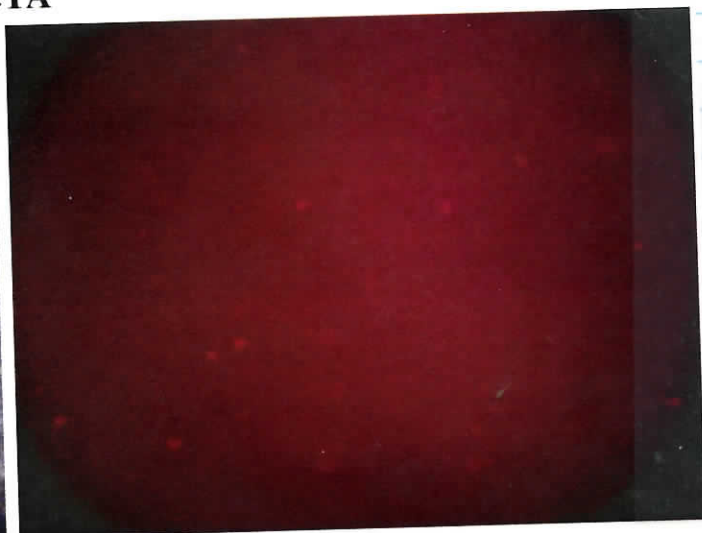
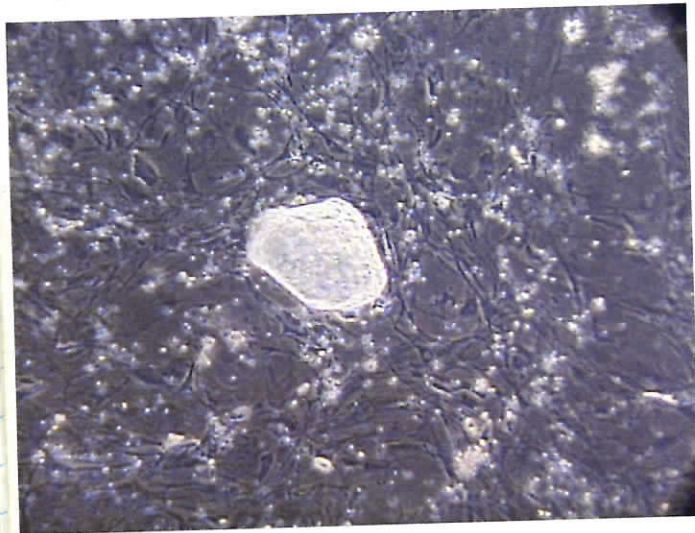
The variegation is interesting

(Y_{CTA}) is reasonably well expressed.
Colony number is okay.

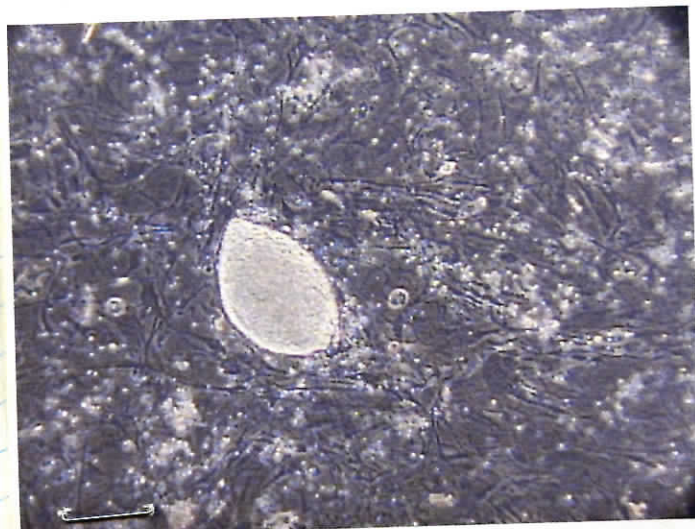
YN_{CTA}



R_{CTA}



MR_{CTA}



OK ✓

YN_{CTA}

Suspiciously as if clones (small number) do not survive if they express YN.

A few individual cells (debris?) express.

Sequencing YN_{CTA} with L'7 CTBlue & K'17 Red Mlu shows both ends are **OK** ✓

165. seq

165. seq (reverse of) K'17 (1 pl)
166. seq L'7 (5 µl/100)

166. seq

OK ✓

R_{CTA}

Intro to YN

Sequencing R_{CTA} with L'7 CTBlue primer & K'17 Red Mlu primer shows that this is **OK** ✓.

161. seq

161. seq (reverse of) K'17 Red
162. seq L'7 Blue

162. seq

Not correct X

MR_{CTA}

Intro to YN. Sequencing MR_{CTA} with L'7 CTBlue & K'17 Red Mlu showed that the 3' end is okay but NheI did not change to AatII → so our AatII site on mito. localizing signal was used. **Not correct**.

163. seq

163. seq (reverse of) K'17 Red

164. seq

164. seq L'7 Blue

L19(2)
CNCTA

L'19 (3)
CN_{CTA}

↖ To Ligo
↙ probably

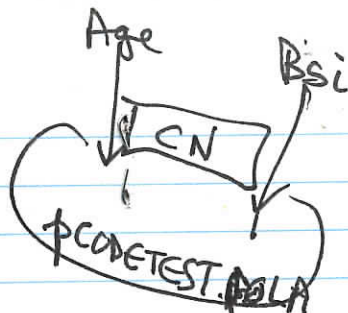
① ~~on~~ ^{2nd} ~~of~~ ^{by} ~~the~~ ^w ~~the~~ ^r ~~the~~ ^z ~~the~~ ⁿ

ect.

CNCTA

Mon 30
Sun. April 29th

CNCTA



19

ably

Take ✓ 5 μ l L'13 (CN) Frag.⁺

✓ 2.5 μ l ddw

✓ 2.5 μ l K145 Age/Bsi (CTA) CAA

✓ 1.2 μ l 10x P95 lig. buffer

✓ 1 μ l T4 ligase K59

15⁰ 6:22 pm to Gld room, 12 pm to

① 2:55 pm
on 4/29/01

Age 1.5 μ l HK2
W TV/cn

DH5d

L'19 L16⁺

09:17a

90 sec 42⁰

8 μ l 65 μ l

5 μ l

to 9:47a

③ ①
R, 20, 5, 20/16, 5/16

1x Amp NZY 37⁰ 9:56am

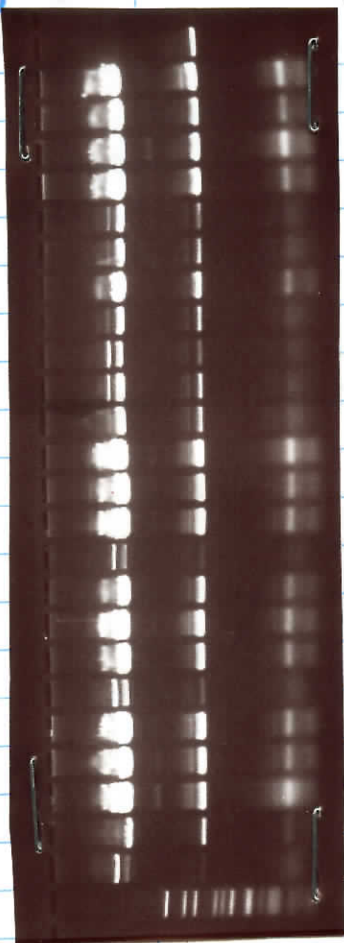
①
②
③
W

Age. mm

W

Age

If the 3 +ve are not correct, repeat with Xh Gold
Tomoko made minus & digested with Age and Bsi WI
TH3-102 digests



① Conclusion: The trouble is
further back. Start again
with Nhe I cut & change
to A&E II using

K'154 Pool
R → Bsi

① 2:1
ON 1/2

8+8

#11
sam
&

A&E II
MIX

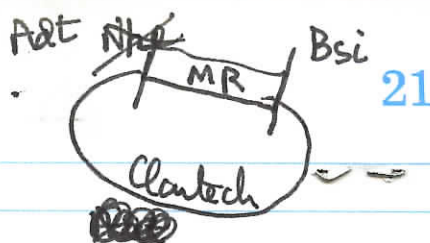
8

1/2

① 2:20 pm
 ② 3:00 pm
 1.5% H₂O 27/10
 RNase 3/10

Sat - May 5th

MR rescue



8+8 K'161 MR frag^t

① Age
 Bsi
 L21

② 24 mins

PCR MM

W

Age

#1 had
 some
 frag. contamination

Seigo's fluorescence of the sequence of MR CTA suggests that the Nhe to Aat did not work properly on K'155. Looking at Clontech data shows that there are already 4 (1) Aat II sites in the plasmid with one at 448 which is close to the beginning of 163. seq. I would explain why whole of 163. seq is homologous to MR sequence.

Go back to K'155 "big" (Nhe → Aat) & retransform

(but has an odd extra label directly on tube of "CTA" - can't be anything but correct)

L'1 PH5α	80 pl
K'155 (B) big [?]	5 pl
00	4:22 to 5:22 pm
90 sec	42°

Plated NZY Kan
 R 20 5 20/ 5 37° 5:12 pm
 many (N20) (16) 9 am man

L'21 KAN NZY Minis ① - ②4 11 am.

Aat II MIX

take 25 pl 10X NEB #4
 175 pl ddw
 2.5 pl (500) Aat II 47 (200 pl) ✓
 80 pl aliquots + L21 minis ① - ②4 11:30 am to 12:30 pm
 2 pl

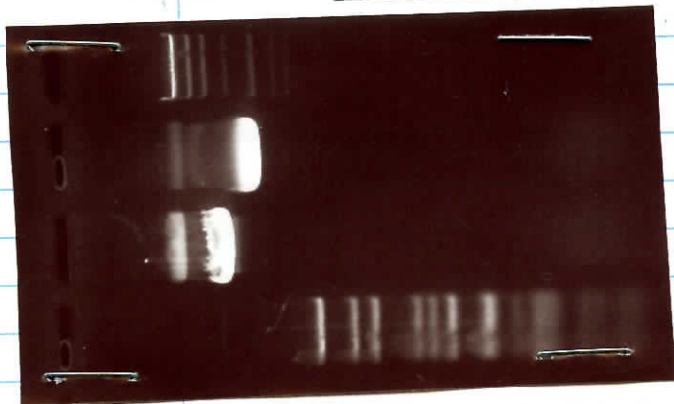
Agarose with Aat & Bsi
 fuse K'161 MR frag^t
 as wrong marker
~~MR frag^t~~

Bsi WI MIX

50 pl 10X RI salts ✓
 200 pl ddw ✓
 5 pl (500) Bsi WI lot 18 (100 pl) ✓
 Add 10 pl to each mini → 55 12:43 pm to 1:52 pm

Fisher 6459

^{melted} ← gel appeared
to have crystals in it!



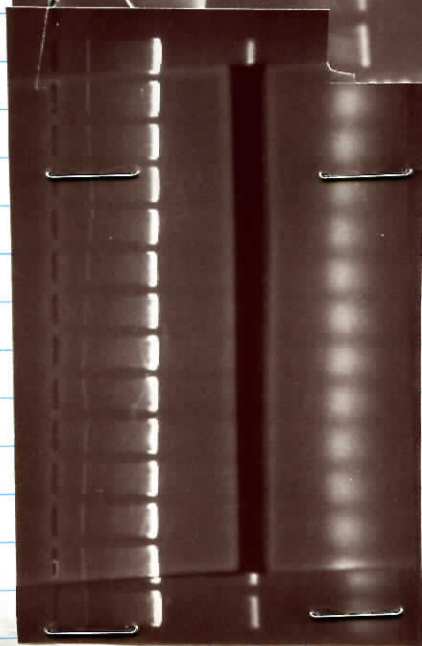
(1) Biggest not quite complete.
Gel was poor (Some debris?).



(2) N he → Aat w/ G.N. All correct.
Pooled at

L122 Pool **MR**
Aat-II / BSIWI

Note: this ^{gel} still shows some entry shadows



(3) products. Proceed.

24



(1A) Cult are good.

Discarded the minus L'25 (1)-(12)

(Error) - sh'd have started with K'138 hr GN(12)
Age / Bsc

(Man)

Take 50 pe 10XRTI 500u
pe ddw

4 pe minig
L'25 (1)-(12)
+ 40 pe aliquot

5 pe (50u) Bsc WI Lot 18 (10u / pe)
25 pe (50u) Age I Lot 33 (2u / pe)
? - L'25 7/12

37° to —
55° to —

Transformation L'100 > DH5α

L'25 hr G

(1)-(12)

AMP NZY Sat
2:30pm - 10:30am Sun

L'25 hr G

0° 2:10 to

90 ser 420

8 pe plated (AMP) NZY

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

pe rhams' (230) (17) (3)

L'25 hr G 37° 14pm

(200) cc

15° 10:30 to 12:50

RT 12:50 to 1:50

37° 1:50 to 1:40

Take 9 pe ddw

3 pe L'25 hr GN Bsc Sac / Bsc CIAA

6 pe hr G L'25 Sac Steps Bsc Rhw oligo

6 pe hr G L'25 Sac Steps Bsc Red oligo

3 pe p95 10X hr G - buffer

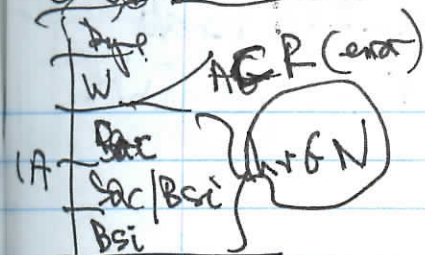
3 pe K59 T4 ligase

L'25
hr G
hr G

1.5% H₂O₂ 10R 10V/c

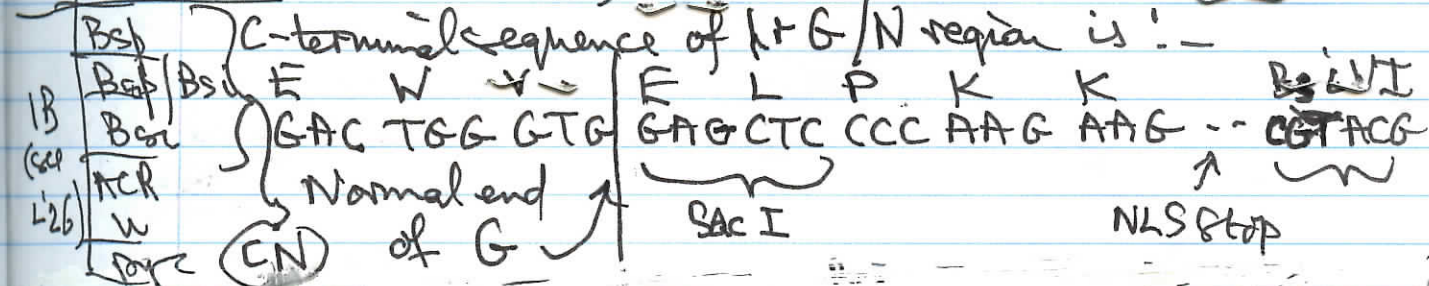


Wed - May 9th



hrGN → hrG (Sac I to Sac I stop ~~stop~~)

Bsi WI



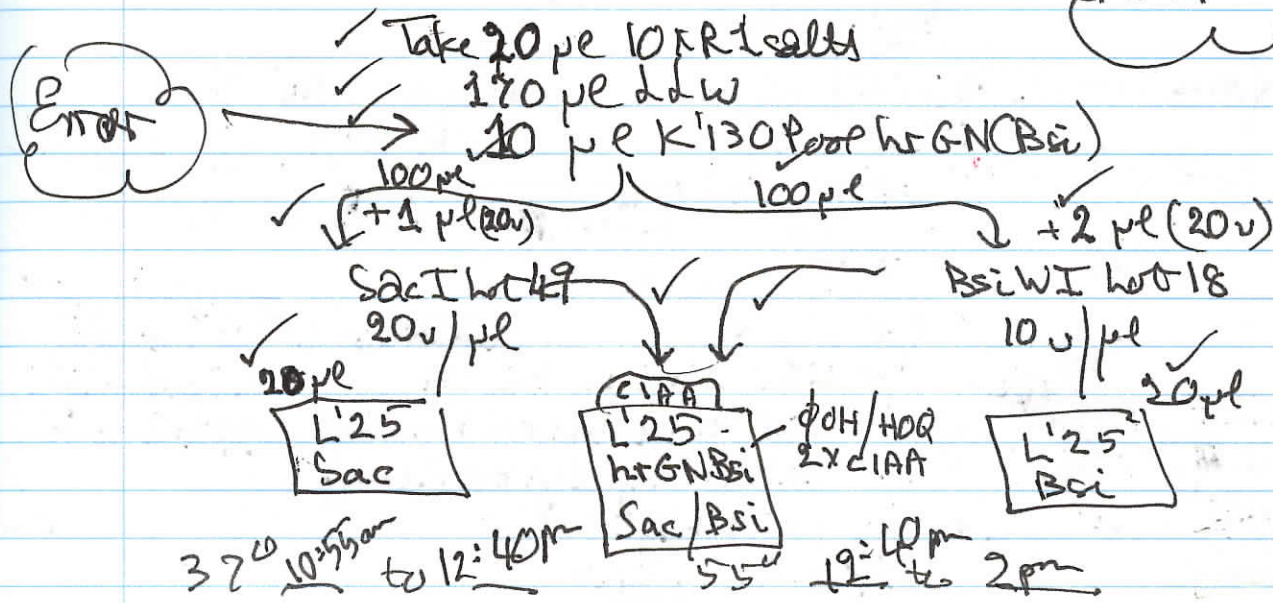
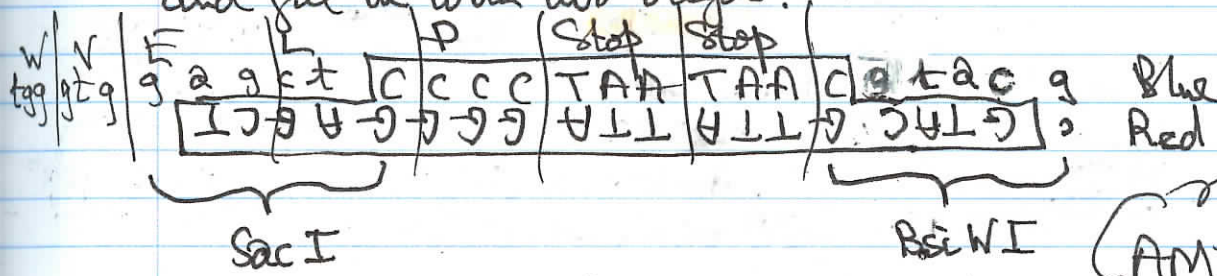
Cut with Sac & Bsi and insert

Use K130 Pool hrGN (Bsi)

hrG 125	Sac Stop	Bsi Blue	
GTA	CGT	TAT	TAG
AGC	T		
hrG 125	Sac Stop	Bsi Red	
CCC	CTA	ATA	AC

AS salts 37° + 55°

Plan is to cut hrGN Bsi with Sac I and Bsi WI and fill in with two oligos:

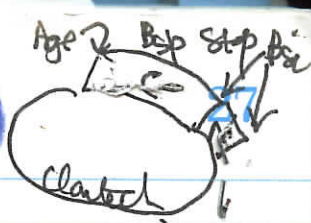


NZY 5/16 3 5/4pm

to 12:50 50 to 1:50 50 to 2:40

01:57
08:23
1.5/10 3/10 RNAse
2/10

Wed May 9th



2

5' H₂L3 (CN) Frag

13 last in cyan

CN → C (Bsp E I to Stop Stop Bsi)

C-terminal sequence of cyan nuclear is: -

23, 24 prep
ACR V Q K S G L R S R ADA K K K R K
W
TGT ACA AAG TCG GACTC TAA TAA TAG NLS

Bsi WI
Cgtacg
3' 5' 195

clonch

Q K S G L Stop Stop Stop
aca aag t CCG GACTC TAA TAA TAG Cgtacg
2 6 8 >> 1 2 3 4 5 6 7 8 9

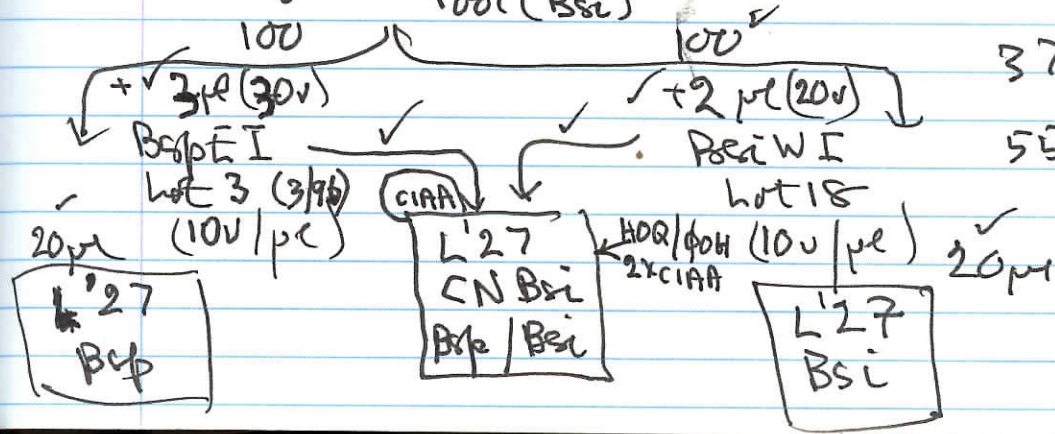
KAN

Results
Cut with Bsp & Bsi
insert

Use K'129 CN
Pool (Bsi)

ligase	5'	3'	Stop	Bsi	Blue
129	CCG	GAC	TCT	AAT	AAT
18	AGC				
449					
129	GTA	CGC	TAT	TAT	TAG
18	AGT				
33%					

Take 20 µl OX RNAse
170 µl ddw
10 µl K'129 CN
Pool (Bsi)



37° 10:55 to 12:40
55° 12:40 to 2m



Seigo will streak
#4 as **MR** CTA (4)
L29

+ use pool for
Zapp

Conce
Mull

Sim

Lu

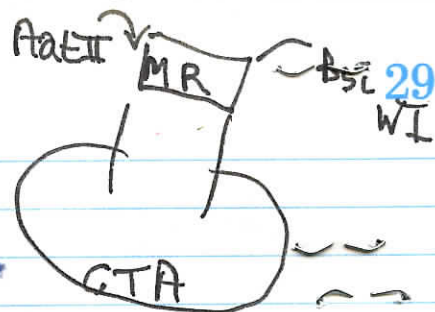
On 9:39

W R
+19 Ki

1
:
:
:
4

MR CTA

Fre. May 11th
R CTA assembly



Take
 ✓ 10 µl L'23(R) frag^t
 ✓ 5 µl K'161² CTA Aat/Bsi CTA
 ✓ 1.9 µl p95 DR lig. buff
 ✓ 2 µl K'59 T4 ligase

L'29
 MR CTA
 LIG^N

changed to MR_{CT}
 Fri
 Cold 4:50pm

Amp

Corrected to L'29
 Nicklabelled
 L'27

Sat. Transformation

Sim Minis ① - ②④
 L'29 R CTA 37° 4:40pm

Processed Mon. - awaiting
 Bsi WI - enzyme for check.

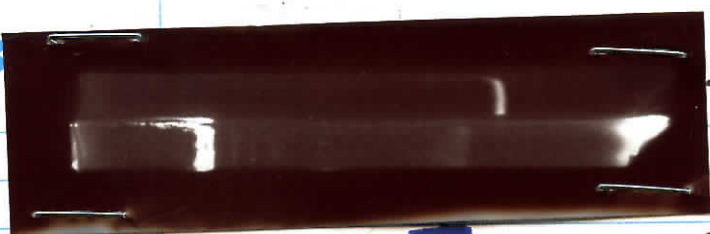
L'25	DH5 d 3/12	Plated 5 AMP
L'29 R CTA LIG ^N	~90 µl	N 27 (16) (6) (0)
2:30 pm	5 µl R, 20, 5, 20, 11, 5, 4	
90 sec	43° 37° 2:45pm Sat	
		10:30am Sun

Take ✓ 26 µl NEB 10X buffer #4
 151 ✓ ~~100~~ µl ddw
 5 ✓ ~~100~~ µl (100%) AatII Lot 47 (200 µl)
 L'29 minis 3 µl to 7 µl AatII Mix
 37° 2:23pm to 4pm

AatII Mix

40 µl/sample
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3



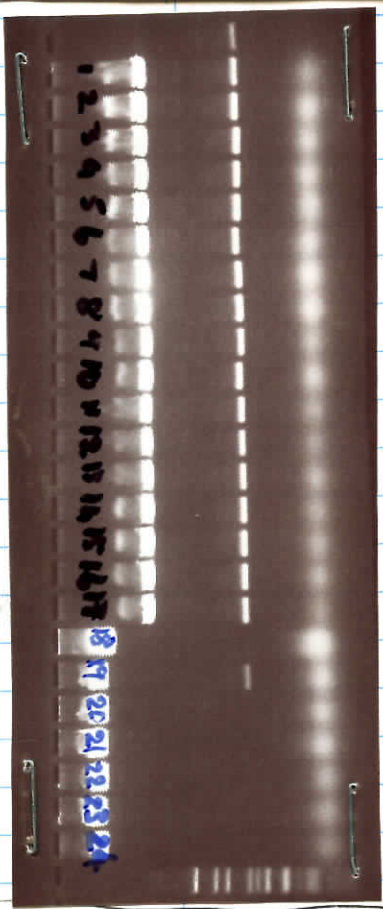
Top
Bottom

ATC 8/11



18

(1) Bsi (old) was ok & Sac.
(C) Fragment is fine.
Gibbs agar ~~still~~ ^{shows} effect (18)
but not with entry problem.



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

(2) Gel on L32 (1)(2), (6) & (10) are hr G-N
Rest are hr G 13/17
Pooled digests for confirmation
on L37

L31 hr G Age Bsi
Pool

minus (1) to (17) to 40 pl Age Bsi mix L33 37' 11:36 am to 12:50 pm; 55' 12:50 pm, 1 to -

hrG

Mon - May 14th

Lr G(repeat)

Diagram illustrating the structure of a protein, likely a histone core, showing the arrangement of amino acids (Amino Acids) and the presence of a histone core (hr G-N) and a histone core (hr G-N). The diagram also shows a histone core (hr G-N) and a histone core (hr G-N).

Age 1

W^{CTA} L'13 Age / Bsi VI

8+8 L33 (C) fragment Take ✓ 20pe 10x R1 salts

L'31 hr G SAC

$$L^1(\mathbb{R}^n) \cap C_c^\infty(\mathbb{R}^n) / B_{\infty,1}^0$$

L31 Kr.G BSc

PCR

W

Page

Take ✓ 20 pr 10x R1
✓ 170 pr 22 w
✓ 10 pr K11

pl K'138 WGN Age / Bci (12)

✓ $\left(\begin{array}{l} 100 \mu\text{e} \\ + 1 \mu\text{e} (20\text{v}) \\ \text{Sac I hot 49} \\ (20\text{v} / \mu\text{e}) \end{array} \right)$

L31
L6
SAC

L'31
hr-GNAge Bsi
Sac / Bsa

✓
 + 2 pc (2 1/2 v) ✓
 BSc WI lot 2
 12 v / pc
 ✓ 1991
 L's
 Mr G
 BSc
 All we have

1xPOH/HOQ
2xCIAA

37° 4:30pm to 5:37pm 55° 6:37pm to 7:05pm (Cold)

231
hw G
LGN

✓ Take bpl & lw

✓ 6 ul 131 I w GNAge Bsi Sac / Bsi CIAA

✓ b' weil G L'25 Sac Stops Bei Blue oligo

✓ 6 µl hr G L25 Sac Rps Seri Red oligo

✓ 3 ne p95 10x 64 - buffer

✓ 3 113 K'59 T4 ligab

✓ 3 p-1 k 5114-492

For gal ~~Shirley~~

Port
L'31:
Sac B3c

15° 3:15 pm to 5:35 pm RT 5:35 F to 7:40 pm 37° A full mounted

Transformer 3/12 D152 80 pF (AMP)

Mimis L31 hr G L31 hr G 5 pF 8:17 p 1 x AMP NZ4

(1) - (17) 0° 4:15 pm R, 20, 5, 20, 5/16, 5/16

1 x AMP NZ4 37° 5:30 pm 90 sec. 42° many (730) (11)



① Good cuts.

211

01/2/65
1.59 HX2
CCTA
7/10

②

Age

5+10

hr G N for

①

L'31

hr G N for

Age

①7

①8

L'33

CCTA

②4

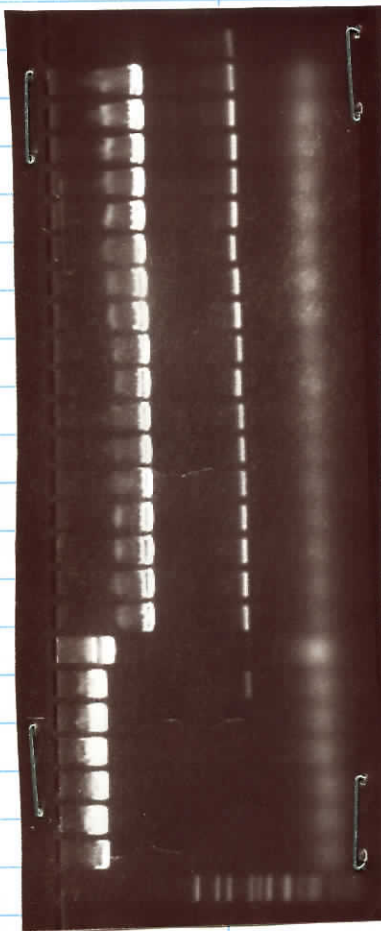
Age

ACK

MM

W

Age



② hr G analyzed on page L'37

← L'33 ①9 in CCTA ; to Sergio

1.59 Hx2
(loop out) R/NH

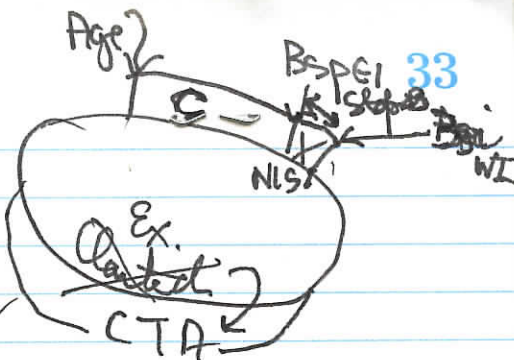
Dye
W
6 N frag: K'38
① L'31
1 CTA
Age Bsi

①7
⑧ L'33
1 CTA
Age Bsi
②4
ACR MN
W
Dye



Mar - May 14th

C (contd.) & CTA assembly



① On 4:45 pm
5:45 pm
0.810 Hx2
3/10 R/NH
5V/cm
Dye
W
14 slots L'26 Age/Bsi
C pool
W
Dye

Processed to 50 µl
as L'33 (C) frag!
Gel on L'30
This is Age/Bsi

✓ Take 7.5 µl L'33 (C) frag⁺
✓ 1.25 µl 10x p95 ~~buffer~~
✓ 2.5 µl K'145 Age/Bsi (CTA) CIAA
1.25 µl T4 ligase K'59
15^h 4 pm to 6 pm RT 6 pm to 7:40 37° A few minutes to

L'33
LIG (CTA)

Transformation

3/12 DH5α 80 µl
L'33 CTA LIG 5 µl 1x Amp 37° 8:30 pm
0^h 1:52 pm to 8:17 NZY
90 sec 42° R, 20, 5, 20/5/16
⑤ ② ① ① ①
(Note 1 per RT, 1 uenz 1, 1 uenz 2)

✓ 85
Take 7.5 µl 10x R/I salt
7.5 µl 6.5 µl ddw
1.25 µl (85) 2.5 µl (85) Age I lot 33 (20 µl)
8.5 µl (85) 2.5 µl (85) Bsi WI lot 18 (100 µl)
16 x 40 µl aliquots for L'31
20 µl aliquots + 2 µl minit ⑧ ②4 37° 11:30 am to 12:50 pm
55° 12:50 pm to

L'33
Age Bsi
mix

To:
Oliver Smithies
University of North Carolina at Chapel Hill,
Department of Pathology and Laboratory Medicine,
CB#7525, 701
Brinkhous-Bullitt Building,
Chapel Hill, NC 27599-7525

May 11, 2001

Dear Oliver,

Please find enclosed the 3.5kbMHC-pGL3 promoter-reporter construct in 20µl 0.5xPBS at ~1µg/µl concentration. Also enclosed the restriction map of the sequence using most popular enzymes.

The 3.5kb MHC promoter is cloned into pGL3 basic (see promega technical bulletins on the web) at PstI site (5') and Hind III site 3'. Pst I is originally SacI on the pGL3 multicloning site. SacI was changed to Pst I in order to make cloning possible.

Thanks for agreeing to share with us your GFP bMHC construct when ready.
Let me know if you have any question regarding the construct.

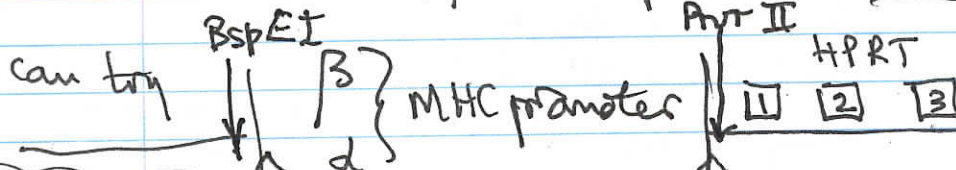
Sincerely,
Fadia Haddad

~ 3 Pst sites,
~ 3 Hind III sites

Can't
Better idea

Fri. May 24th

For the α & β MHC promoter selection experiment



p5KB1 ~~XXXX~~ NOPRO

J99/23

Use (AVR II) NOPRO instead

29002

L34 Kpn Age Kpn

CCG	ACC	GGT	CGG	GTA
16	C			
75%				

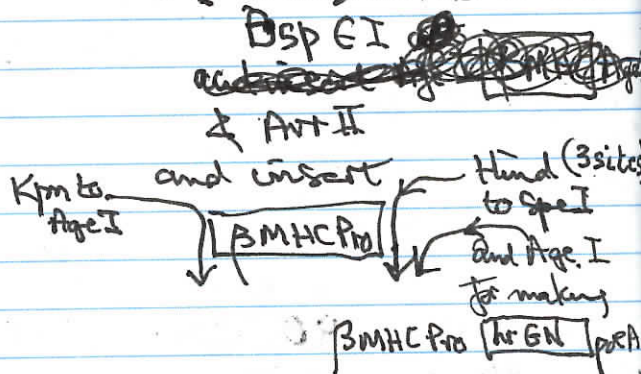
BspEI

29003

L34 Hind Spe Hind

AGC	TTC	CAC	TAG	TGG
16	A			
50%				

for Avr II



29004

L34 Hind Age Hind

Age I

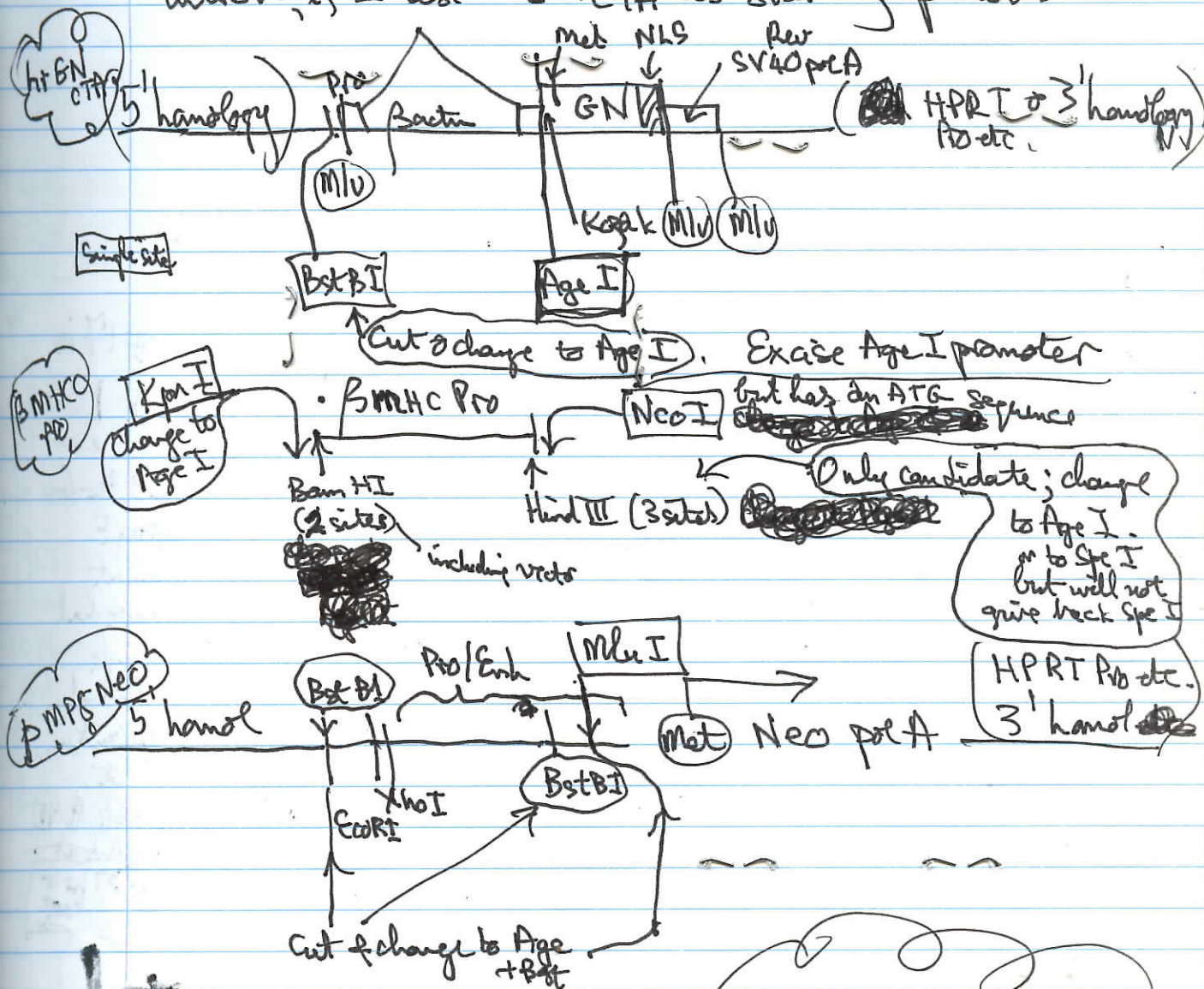
Hind HI

L34 Hind Age Hind

AGC	TTC	CAC	CGG	TGG
16	A			
63%				

Can't on L53

Plasmid rec'd from Fabio Haddad. First job will be to replace β actin promoter with this promoter & test in ES cells to cardiac myocytes. Simple construct will not have an intron, if I use GNCTA as starting point.

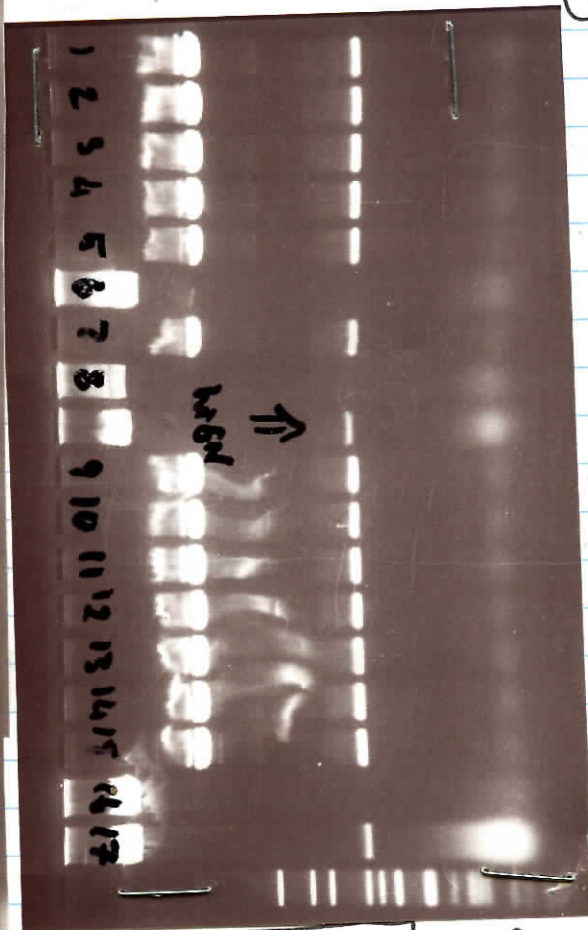


(Continued on p. 34)

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
GN	GN	G	G	G	GN	G	G	G	GN	G	G	G	G	G	G	G
X	X	✓	✓	✓	X	✓	✓	✓	X	✓	✓	✓	✓	✓	✓	✓



DA separate cuts.



(2)

#17 is
X!

L37hr G
fragt.
must
have had
single
strand
parent in
it.

①-⑤⑦, ⑧-⑫
Pooled at
12-27 hr G
pool

← !

L37 #17
hr G CTA

to Seigo for
sequencing growth

to G

Thurs. May 17th
hr G (contd.)

hr G ϵ TA

37

L'31 hr G Age Bsi Pool

Sat Gel from L'32 shows 24 bp decrease in ϵ TA expected with G (stop) versus GN (stop). Pooled appropriately. Isolate & clone into CTA. Red K'17 Minis sequence to confirm.

Recovered into 50 μ l as L'37 hr G fragt.

Age w/ Scrap (1)
L'31 hr G Age Bsi Pool

14 ϵ TA
1.5 μ l H₂O x 2 W 3/10 RNAse 7V

✓ Take 7.5 μ l L'37 hr G fragt
✓ 1.25 μ l 10x p95 lig. buffer
✓ 2.5 μ l K'147 Age/Bsi CTA
✓ 1.25 μ l TA ligase K59

Sat 150 4:55 pm to 27th RT 27th to 8:28, 37^o 8:28 to 8:36 pm

L'37 Age Bsi
hr G CTA

Transformation

1x Amp NZY

Sat

hr GN K'147 (5)

3/12 DH5 α

80 μ l R, 20, 5, 20/16, 5/16

37^o 9:08 pm

L'37 Age Bsi
hr G CTA

L'37 hr G
lig- CTA

5 μ l (8) (7) (2) (0) (0)

1x Amp NZY

90 sec 42^o Mon

8:58 pm

L'37 hr G CTA

1x Amp NZY 37^o

12:45 pm

1x Amp NZY 37^o

Take 40 μ l 10x R I salts

8:45 am

20 μ l + 2 μ l L'37 minis (1) - (17) plus K'147 2/153 hr GN (5)

336 μ l ddw

20 μ l (40u) Age I lot 33 (20 μ l)

hr G CTA

4 μ l (40u) Bsi WI lot 18 (10u μ l)

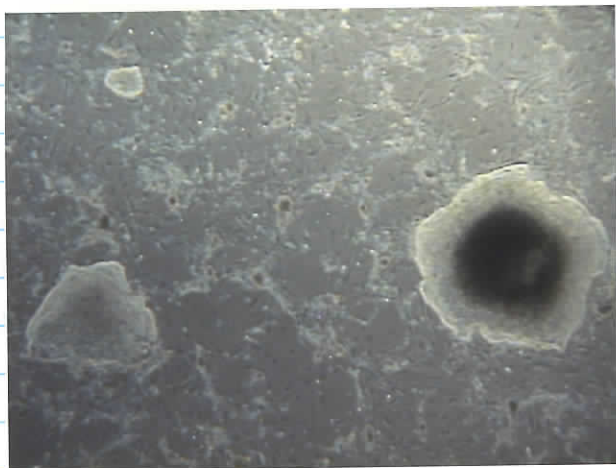
37^o 12:25 pm to 1:33 pm

2:20 pm

for growth

37^o 12:25 pm to 1:33 pm

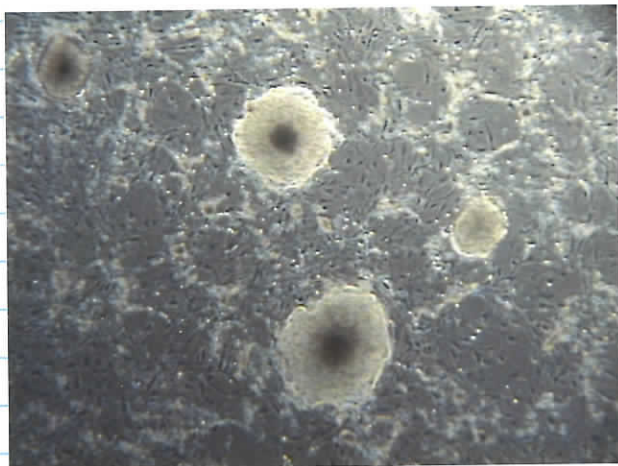
38



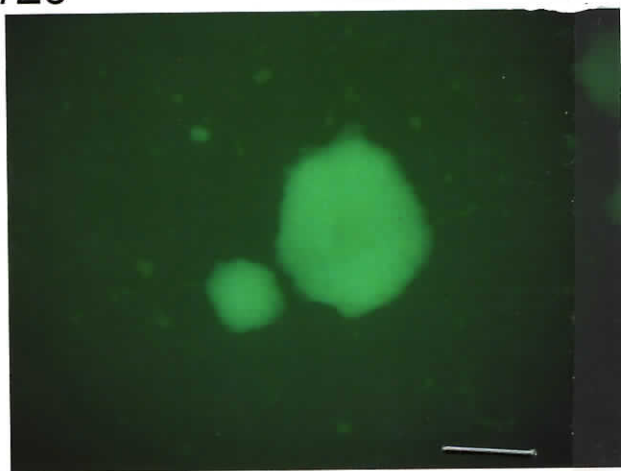
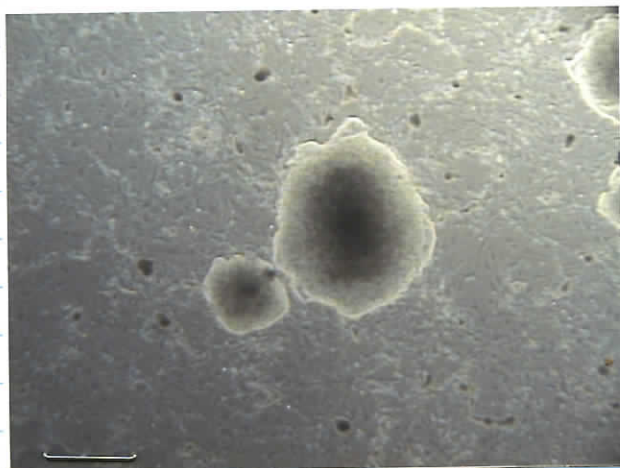
138



138R

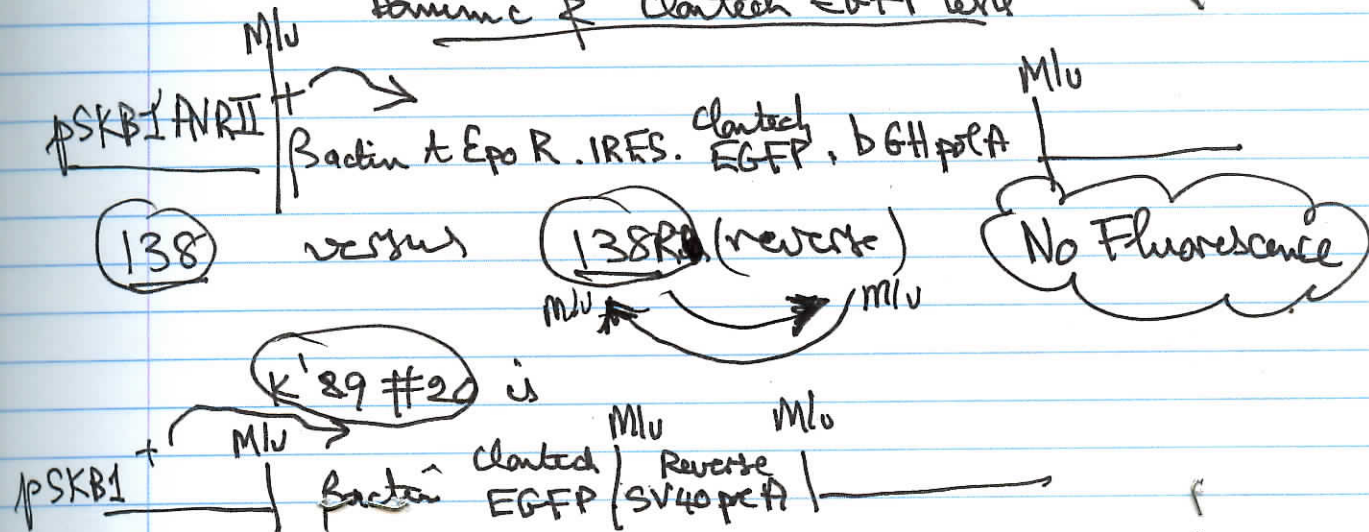


K'89#20



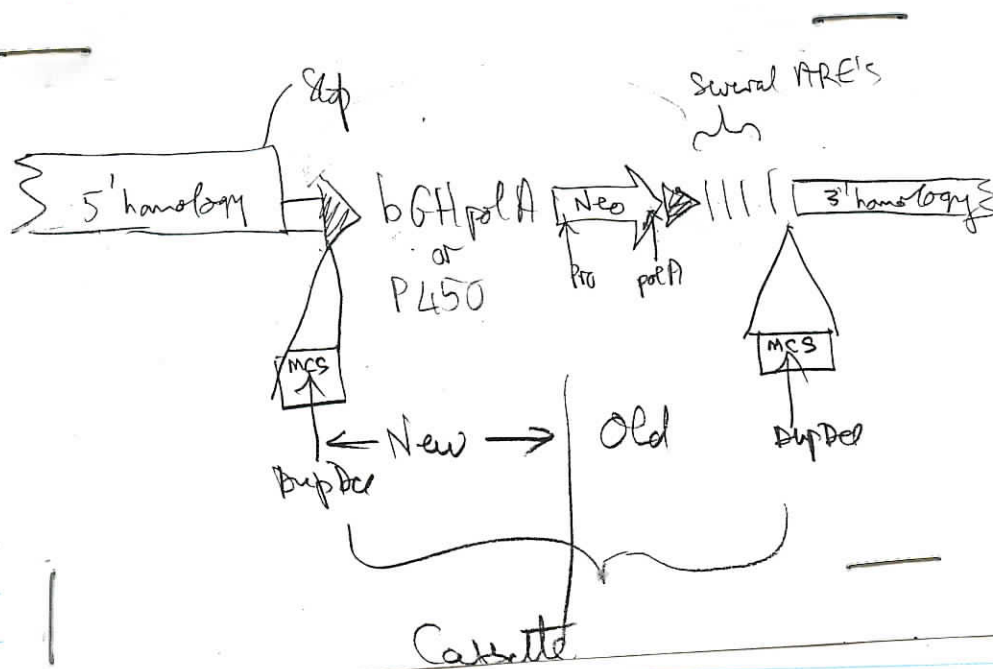
Tues. May 22nd

Dominic & Clontech EGFP tests



Conclusion: both 138 & 138R give some residual non-colony fluorescence, but no colony fluorescence, & no effect of reversal.

(K'89 #20) (G_{CTA}) is working fine.



Tues. May 22nd

NM/OS HILO targeting vector

Convert OS DUPDEL
to
OS HILO

Use without Cre to get High expression
Use with Cre to get low expression

→ 1/6 hormone or P450 3'UT.
→ 3'UT with multiple ARE's.

CTA

Tues. May 22nd

Sequencing data

MR CTA

43

168.seq

169.seq

L'7 CTA Blue primer
L'33 (19) **CTA**
K'17 Mlu Red primer

(reversed sequence)

Age I to Met 7
on is correct for CTA
~~5' identical to~~
CODEST. POLA 4 to 17

Mlu to Mlu 3' Rev
PolA is correct.
linker with (stop) is
fine to end cyan FP.

170.seq

171.seq

L'29 **MR CTA**
#4
L'7 CTA Blue primer
K'17 Mlu Red primer

(reversed sequence)

5' identical to
CODEST to 26.
Identical to MR
from 27 on, including
Met ATG. (Att II is OK)

Mlu to Mlu 3' Rev
PolA is correct.
Usual UAA sequence
indicated where UAG is correct
for 3' end of MR

172.seq

173.seq

hrG CTA
#17

L'7 CTA Blue primer

K'17 Mlu Red primer

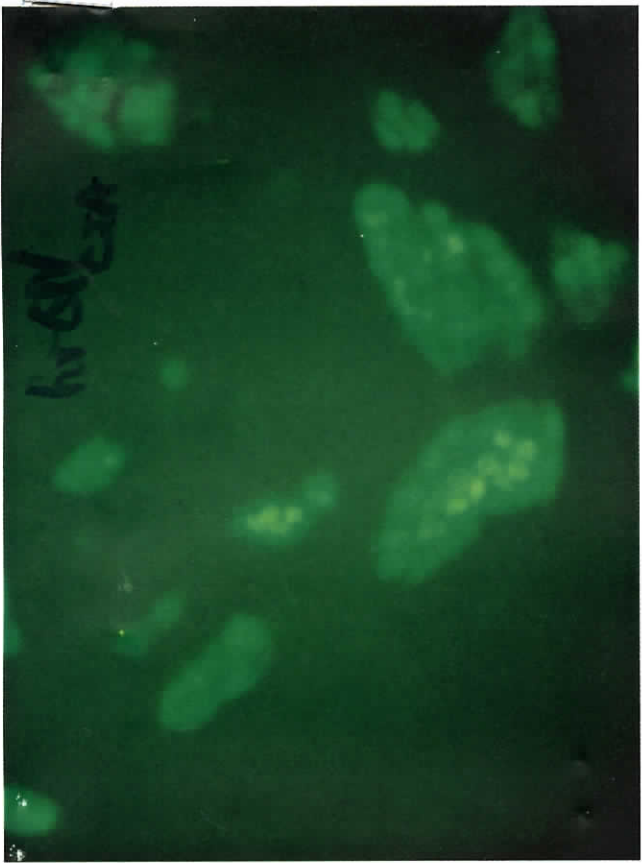
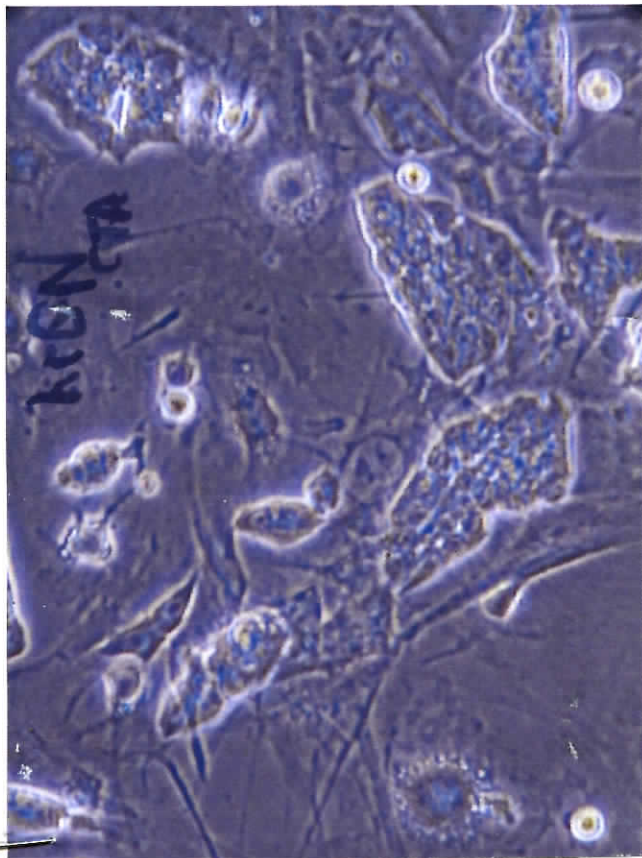
(reversed sequence)

linker to Met
on is correct

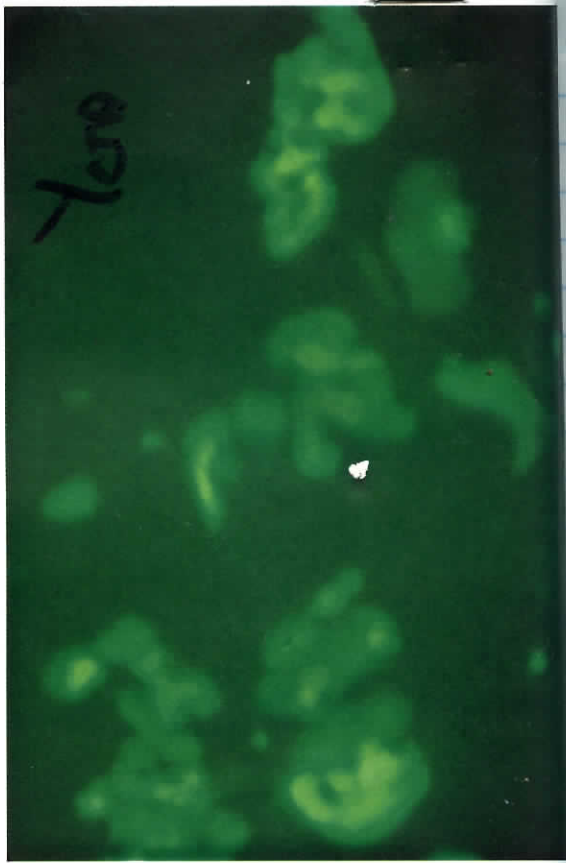
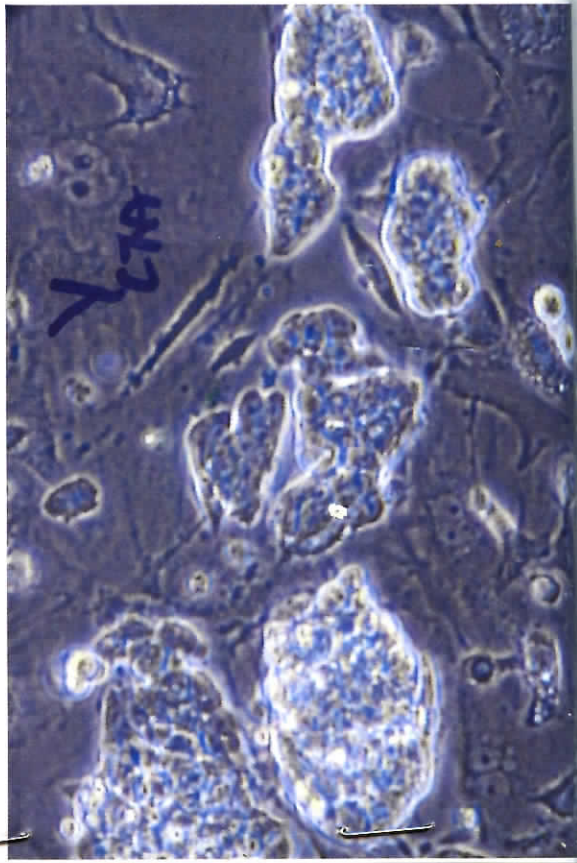
3' end of hrG
with 2 stops
is okay
Mlu to Mlu
checks with
CODEST. POLA

4

hrGN, in regular medium, derived from single colony, +P8, x40 lens



YCTA, in regular medium, derived from single colony, +P8, x40 lens



Wed. May 23rd

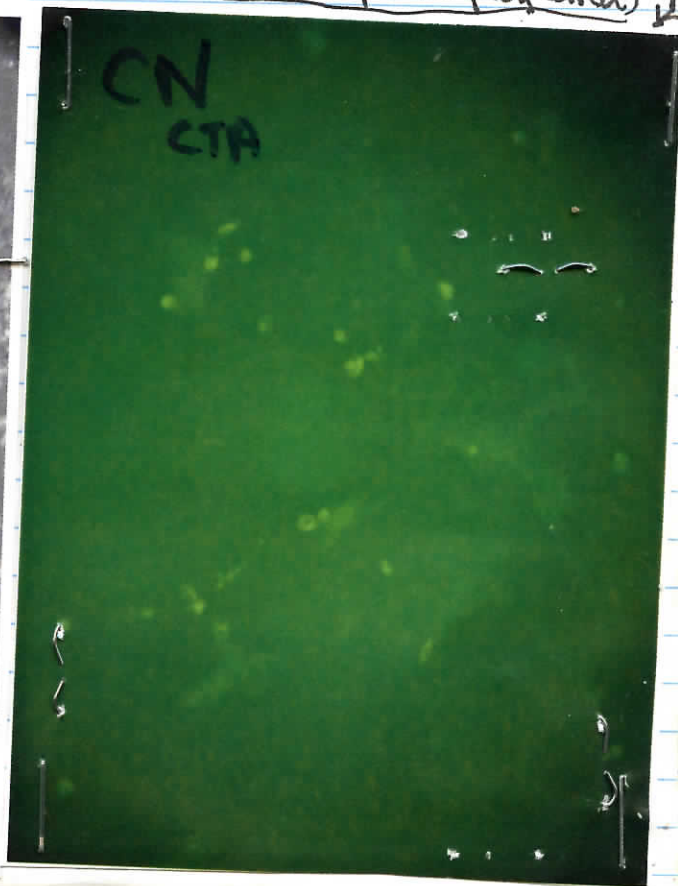
↳ GN_{CTA} cells & colonies from a single colony appear to be completely stable. Nuclear localization varies.

Y_{CTA} little show that some colonies may have become fluorescence-negative.

CN_{CTA} is showing HAT^R colony shut down.

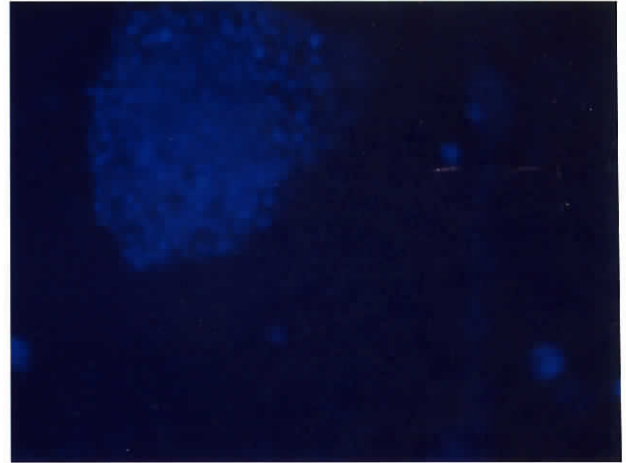
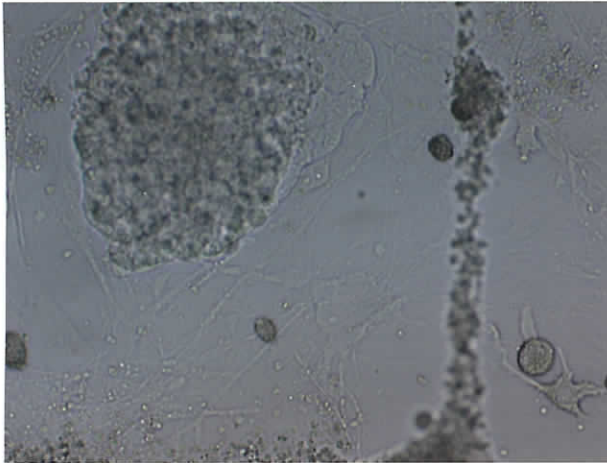
When correct is used -
there is fluorescence.

↳ 6 days after zapping in HAT selection (wavelengths not fully correct)



Thurs. May 24th

CNCTA, in HAT selection, 7 days after zapping, using Nikon microscope, x10 lens
(exact cyan filter)



(rhodamine filter)

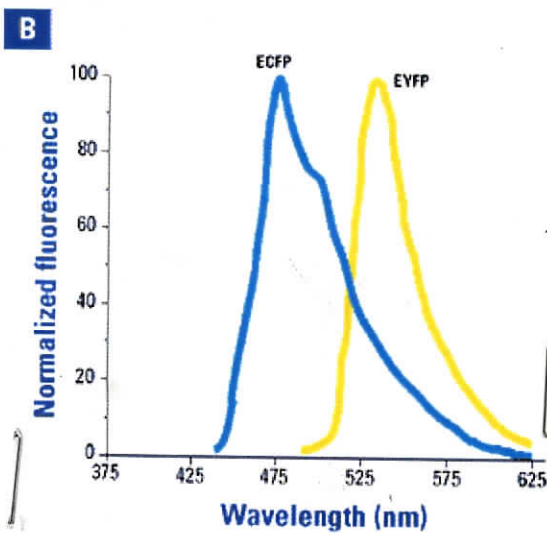
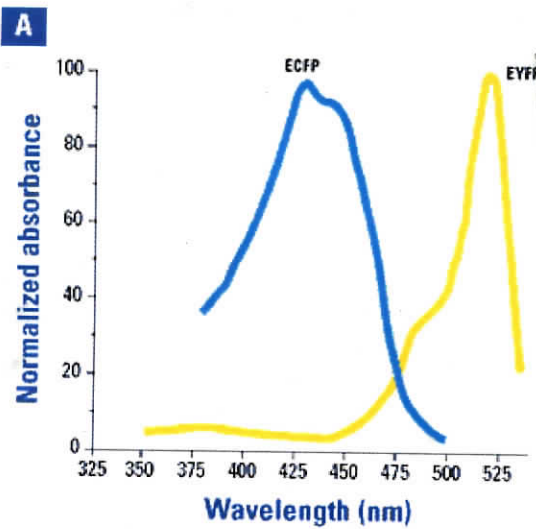


Figure 1. Fluorescent excitation (A) and emission (B) spectra of ECFP and EYFP. Data provided by G. Patterson & D. Piston, Vanderbilt University.

CNCTA
rhodamine filter
can be used for dead cells

Friday May 25th

Intensity calibration

It is clear that cyan nuclear is working well, but at low efficiency. Nevertheless it may pay == to have discriminating wavelengths that are well separated.

Filter Set **XF104-2**

YELLOW

red

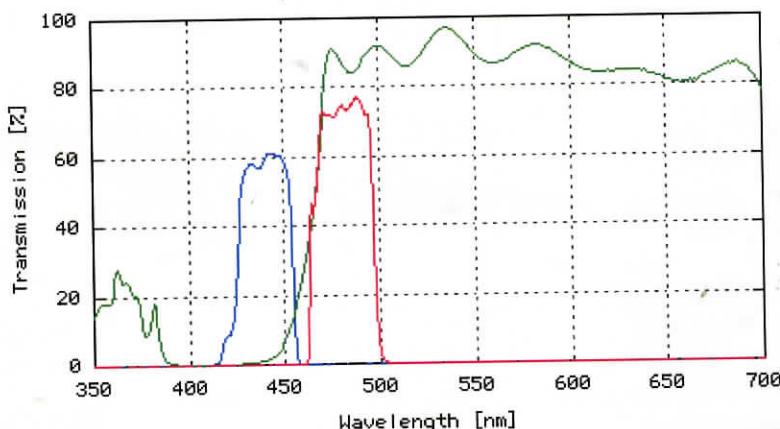
cytoplasmic make

OMEGA OPTICAL incorporated

Filter Set **XF114-2**

CYAN

Since green is a better cyan & clear. The ex well.



min 350 nm

max 700 nm

Showing **XF114-2**

Exciter: [XF1071](#) (440AF21)
Dichroic: [XF2034](#) (455DRLP)
Emitter: [XF3075](#) (480AF30)

Notes: This set is our XF114-2 option, newly revised in October 1999 to incorporate ALPHA technology. The new set has a better signal to noise ratio than our previous XF114 set. This set features a pale blue signal with a very black background. It provides excellent spectral separation from YFP. See XF130-2 for a CFP longpass filter set.

☒ **Fluorophores used with XF114-2**

BO-PRO(TM)-1
 BOBO(TM)-1
 CFP (Cyan Fluorescent Protein)
 Chromomycin

Show All Fluorophores

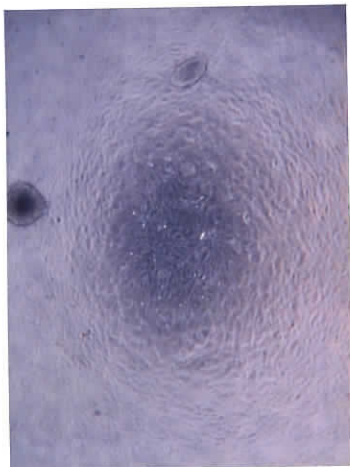
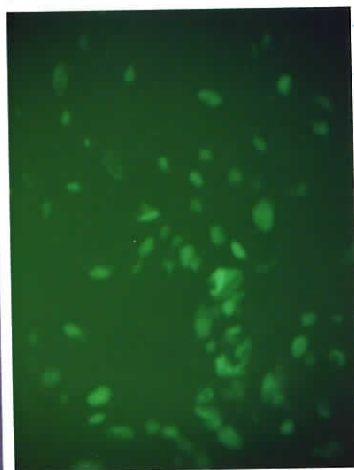
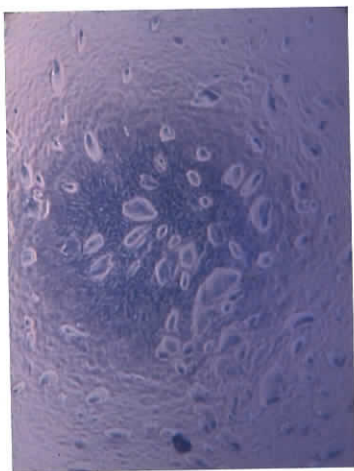
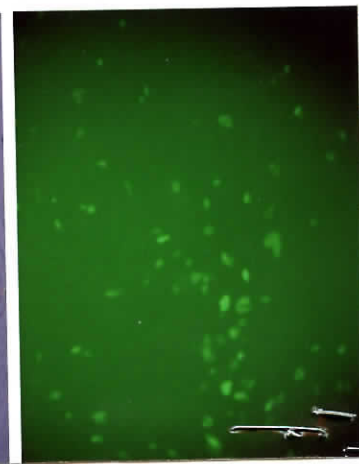
ECFP, E. coli DH5α, the pEYFP, or the observed under filter sets specific for observed with ECFP. Same field observed 04. Panel C. Double uentially.

Sorted EGFP-expressing ES cells after electroporation

3-day sorting

No HAT, day 3

In HAT, day 11

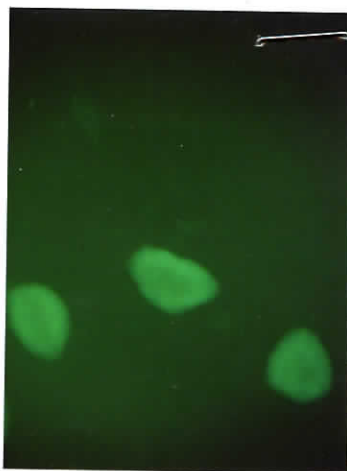


Friday, May 25th

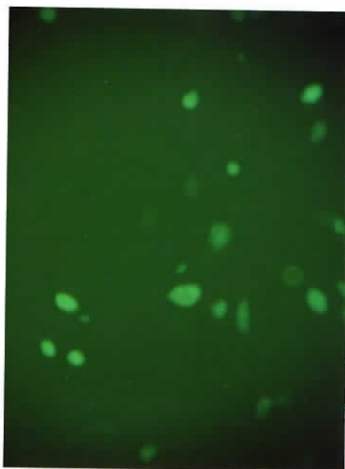
Cloned EGFP survival selection
etc. well. The transient &
random integrants are easily seen in these
sorted cells.

7-day sorting

In HAT, day 12

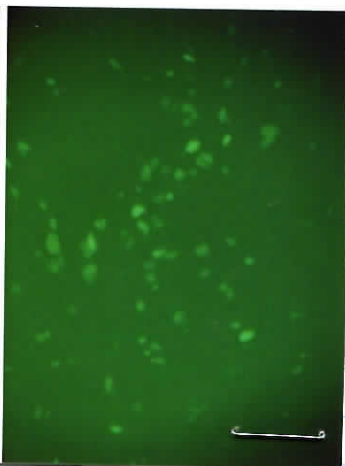
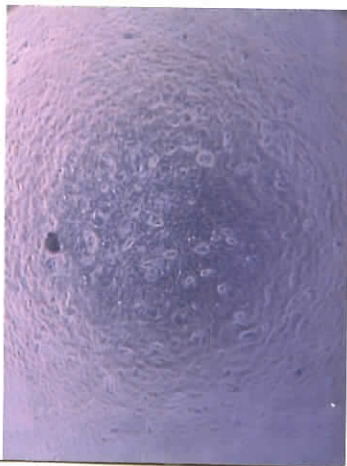


No HAT, day 4



First day sorting

No HAT, day 1



Fri. May 25th

Nobuyo's (TG) (AC) hr GN CTA's

Take
 ✓ 4 μ l K¹⁴⁷ hr ~~5~~ 5 (hr GN CTA)
 ✓ 4 μ l 10x RT buffer
 ✓ 28 μ l ddw
 4 μ l (4 v) Age I hot 33 (20/ μ l)
 37° 2:53^{pm} to ~~5:26~~ 5:10^{pm} → 10 min at ~80°C

L'51
hr GN CTA
Age

Take
 ✓ 8.6 μ l (2 μ g) L'51 (TG)_n Blue
 ✓ + 3.7 μ l (2 μ g) L'51 (AC)_n Red
 + 1.4 μ l 10x P95 buffer

Blue
 L'51 (TG)_n NM's formula
 0.232 μ g/ μ l
 Red
 L'51 (AC)_n 0.544 μ g/ μ l

To 80°C & allowed to cool from 5:26^{pm} 80°C 0 min
 5:31^{pm} 61.5°C 5 min
 5:36^{pm} 52.5°C 10 min
 5:41^{pm} 47.5° 15 min
 ↑
 Go higher next time...

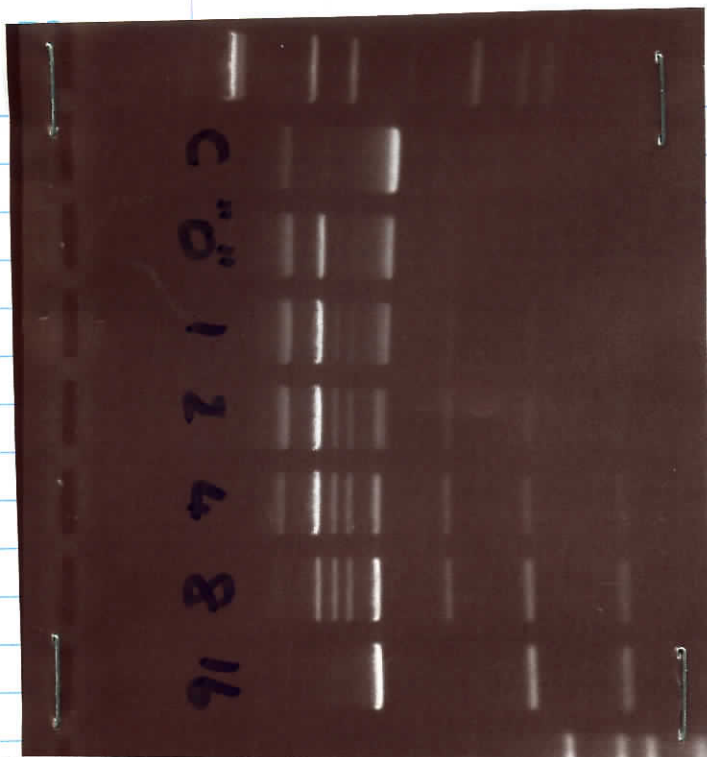
✓ + 6 μ l L'51 hr GN CTA Age (post heating)
 ✓ + 4 μ l ddw
 ✓ 1.4 μ l P95 (10x) liq. buffer
 ✓ 3 μ l K⁵⁹ T4 ligase
 15° 5:47^{pm} to 8:12^{pm}
 37° 8:12 to 8:45^{pm}

L'51
LIG^N

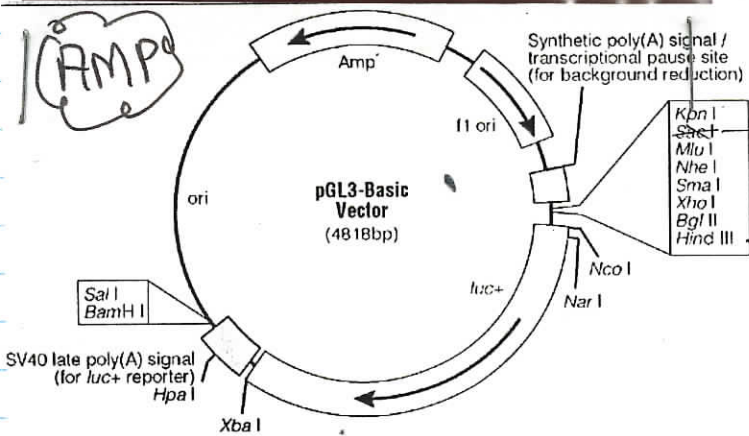
Transformation

3/12	AT5 d	80 μ l	1x AMP NZY	37° 9:13 ^{pm}
	L'51 liq.	5 μ l	R, 20, 5, 20/16, 5/16	
	0° 8:45 to 9:04 ^{pm}	9:04 ^{pm}	Many	(N15) (N10) 11:15 ^{am}
	90 sec 42°C			

None of first 24 were correct.

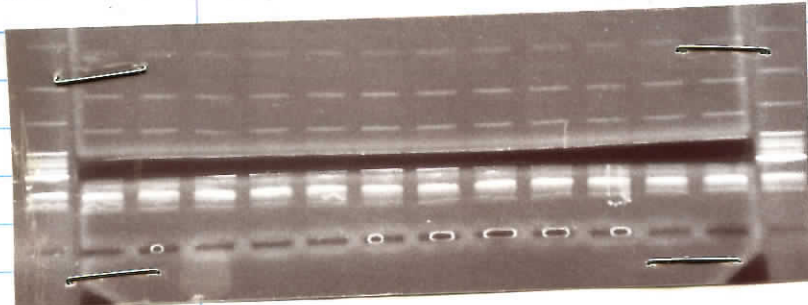
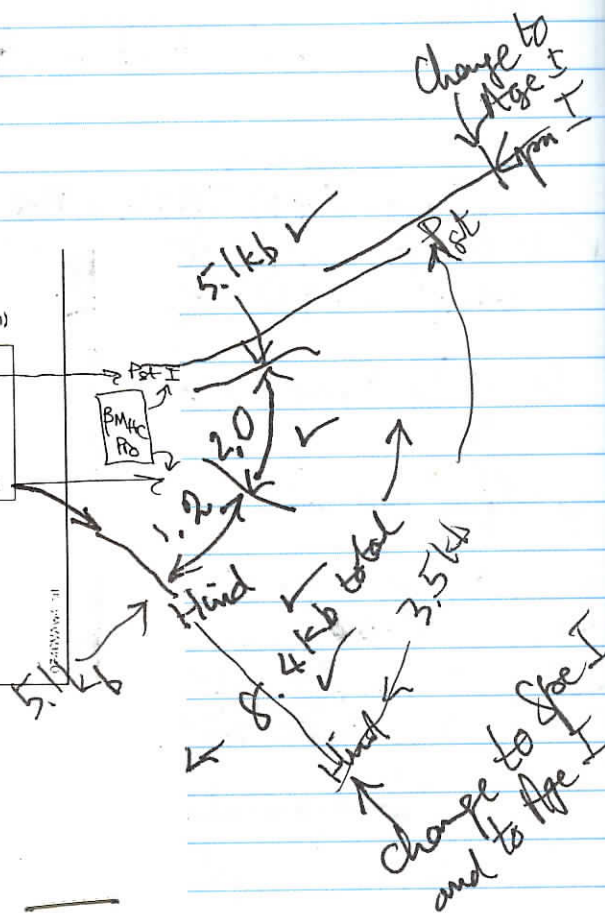


Conclusion - Good series
 Pooled "0", 1, 2, 4 & 8
 as L'53 Pool
 and saved L'53 16



pGL3-Basic Vector sequence reference points:

Base pairs	4818 bases
Promoter	(none)
Enhancer	(none)
Multiple cloning region	1-58
Grimer2 binding site	111-89
Luciferase gene (luc+)	88-1737
SV40 late poly(A) signal	1772-1993
RVprimer4 binding site	2080-2061
ColE1-derived plasmid replication origin	2318
beta-lactamase gene (Amp ^r)	3940-3083
upstream poly(A) signal	4073-4527
RVprimer3 binding site	4658-4811
	4760-4779



(2) As good as can be expected.

series

28

6

to
age I
age I

Spec I
Age I

ected.

on 12:42pm
off 1:42pm
0.8% Hx4
max

AgMM
Control
"0"

1
2 Should have used 400 pl!

4
8
16
ACRMM
W

on 6:04pm
off 8:04pm
0.8% Hx4
20 pl

14
L'53 Pool

Recovered into 50 pl
L'53 Fragt

L'53
L'16"

Transformer

3/12 DH5 α
L'53 lig.
4:43pm to 5:15pm 0°
May 30 Wed 5:25pm 1xAMP NZY
R, 20, 5, 20/16, 5/16

Sat - May 26th

BMHC Pro / GL3 (contd. from L'34)

Sample cut Hind for Spe (and Age)

Take
35 μ l 10x RI buffer
309 μ l L2W
6 μ l (~6 pg) L'34 BMHC GL3
Warm to 32°
Add 0.6 μ l (12 u) Hind III Lot 52 (20 u/pl)

"0", 1, 2+, 4+, 8, 16, to $\geq 80^\circ$ for ≥ 20 min
60 μ l
40 μ l
Rest

Ligation

15 μ l L2W
Take 3 μ l L'53 Fragt (single Hind cut)
3 μ l P95 10x lig. buffer
6 μ l L'34 Hind Spe Hind oligo
3 μ l K'59 T4 ligase

15° (1:30 am to 2:15 pm) RT to 3:27 pm 37° to 4pm

L'53² Wed. May 30th
1xAMP NZY 37° 5pm Sun.

1xR, 20, 5, 20, 5/
Many (10, 16)
L'53 (240) (12)

Minus 1xAMP NZY 37° 4:30pm
D-24

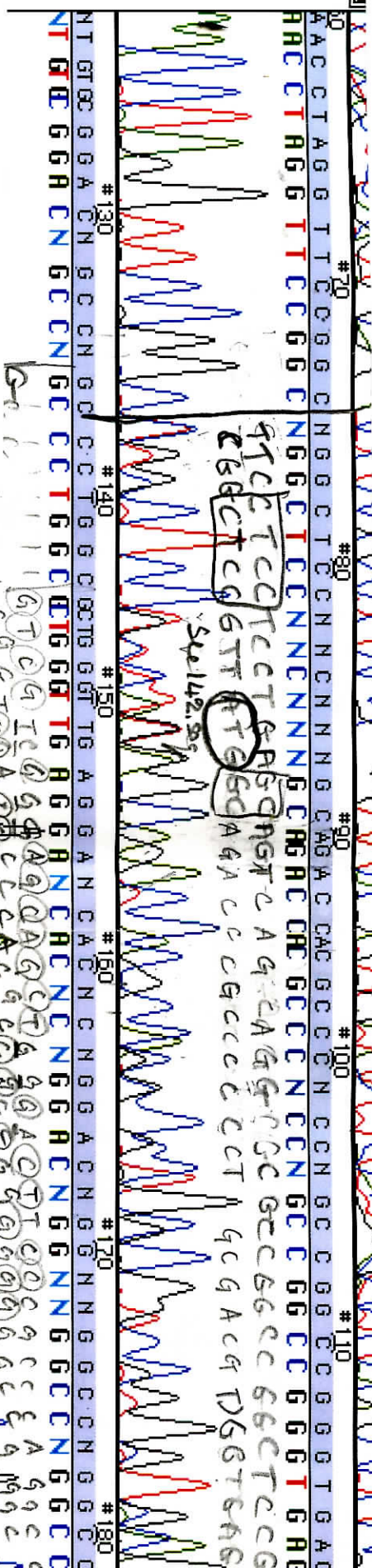
(Continued on L'61)

1 BMHC Pro
Spec I
Cor Age

10007 TAGCTCAAGTGCAGCTCCGCTCCGGGTTCAAGTATCTCTGCTC 10056

51 AGCTTCGGAGCTAGTTC 71

10057 AGCTCGGAGCTAGTTC 10076



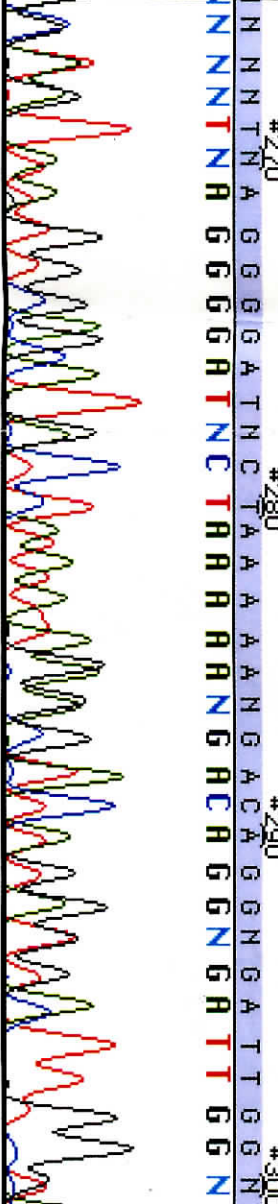
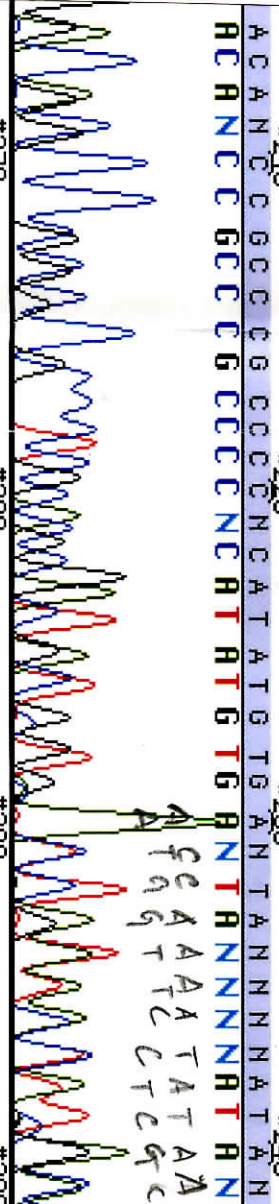
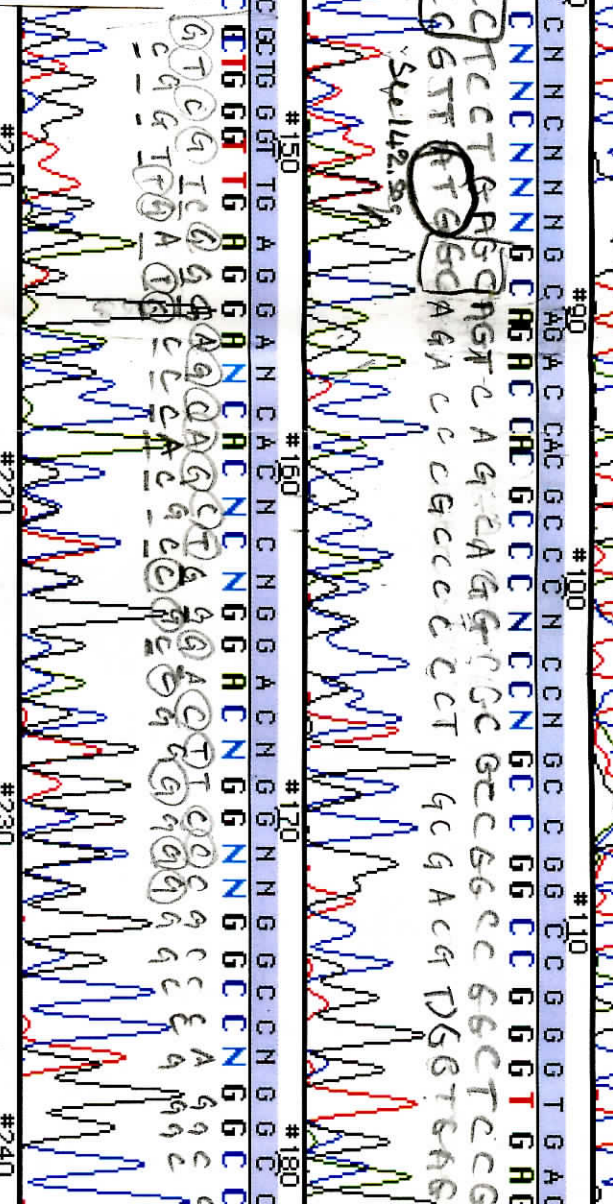
174.seq
/afs/isis.unc.edu/home/n/o/nobuyo/oliver/PSKBLCODETEST.POLA check: 4106 from: 71 to: 171
0253 to: 18518
ASSEMBLE February 28, 2001 12:42
Gaps: 2 Quality: 330 Ratio: 3.438 Score: 18 Width: 5 Limits: +/-6

71 CGGCGGCTCCNNCCNCCAGACAGCCGCCGCCGCCGGGAGCA 120
10257 CGGCTTCTCTCTCTGAGCAGTC..AGCCGCCGCCGCCGGCT..CCG 10302
121 NTGTGGGAGCCGCCCTGGCGCTGGGTGAGGACACNCGACNG 170
10303 TTATGCCAGCCGAGCCCTGGGCTGGTGGAGAGCTGGCGCTGCCG 10352

174.seq
/afs/isis.unc.edu/home/n/o/nobuyo/oliver/PSKBLCODETEST.POLA check: 4732
0287 to: 18518
ASSEMBLE February 28, 2001 12:42
Gaps: 3 Quality: 317 Ratio: 3.562 Score: 16 Width: 7 Limits: +/-8

71 CGGCGGCTCCNNCCNCCAGACAGCCGCCGCCGCCGGGAGCA 120
10291 CGGCGGCTCTGATATGGC..GACCCGAGCCCTGGCGTGGTGAACA 10338
121 NTGTGGGAGCCGCCCTGGCGCTGGGTGAGGACACNCGACNG 166
10339 ..GCTGGCGCTCTGGCTGGC..GGTTTCAAGGCTCACGCGGCA 10379

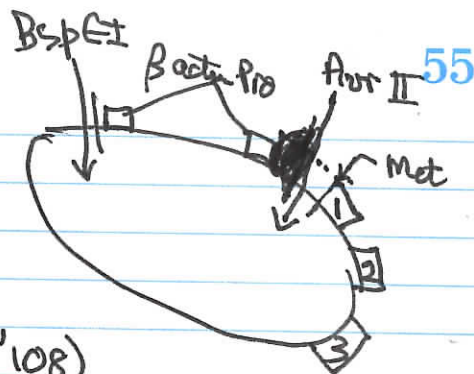
Handwritten notes: (180) delct, difference 34, 10257, 136, 10318, 10352, 10291, 10379, 34, 34.



Handwritten notes: 174, 5'99(26)Base in, Can get the, volume note.

Sun. May 27th

J'99 (26) Pool pSKB1.NOPRO



Take 20 pl J'99 (26) Pool (see J'108)

✓ 40 pl 10x RIsalts

✓ 340 pl ddw

~200 pl ~200 pl

✓ 4 pl (40) BspEI
Lot 3 (100/pl)
(3/96)

L'55
Bsp

37° 3:52 pm to 6:35 pm

L'55
Bsp/Avr
NOPRO

✓ 10 pl (✓) Avr II
Lot 29 (4 u/pl)

L'55
Avr

φ04/HOQ 2XCIAA

Bsp^{EI} will accept Age I; Avr II will accept Spe I; need to φ04 since Avr cuts βMHC Pro & is heat resistant. Single, the two sites each leave 2 bases extra from 6 base recognition. Should be just about ok according to N&B.



Diagnosis of success will be easy. If any problem, use Age βMHC Age

Logical Error

See L'57

Sequence check shows that Pool is a mixture of 2 J'99 (26) Pool with Blue primer

174 .seq

Can get these two out when necessary.

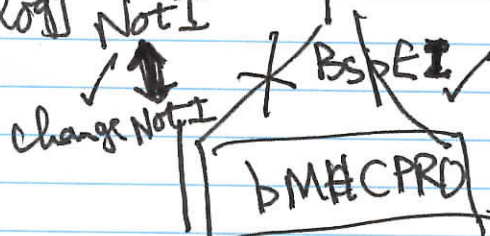
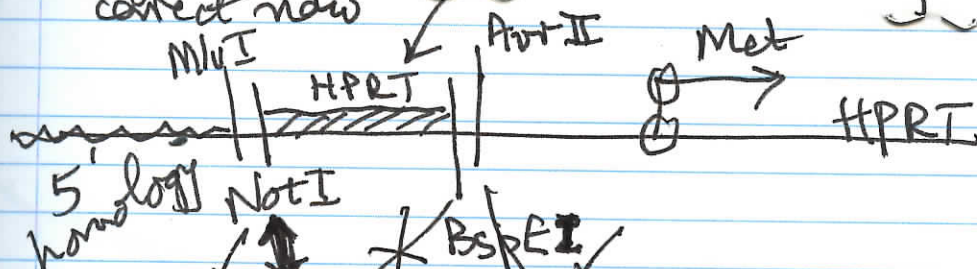
214 bp & 2180 bp deletion of pSKB1

CODE TEST, POLA

Sun. May 27th

Redesign pSKBI. bMHC PRO

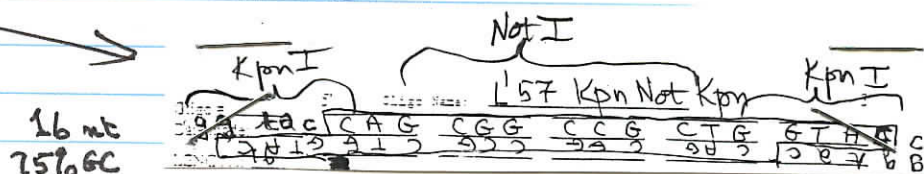
Assembling the proposed construct shows that there are ~900 bp of human HPRT sequence left 5' to the bMHC PRO. Probably better to correct now



Intended Age I Spe I from Hind III

∴ Change (Kpn to Not I) (or Mlu I); Mlu can then remain in pSKBI.bMHC PRO

Cancelled

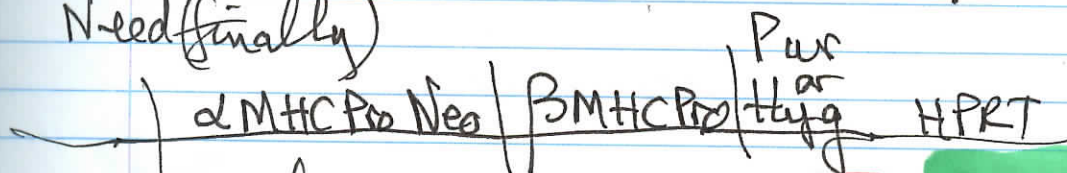


Insert as shown

Error

Faulty logic! Without HAT selection for targeting, too few cells will be seen!! N.M.

Need finally



then

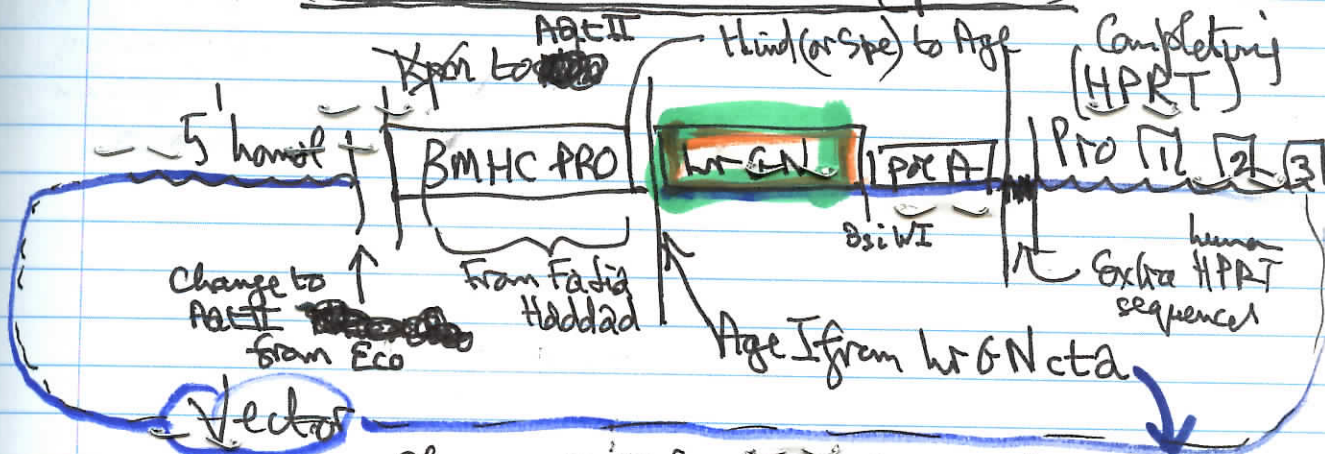


158 Kpn Aat Kpn					
29008	CTA	GAC	GTC	TAG	GTA
16 Kpn	C	Aat			Kpn
50%					
Eco 158 Eco Aat Eco					
29009	AAT	TCT	AGA	CGT	GTA
NOTH 16	G			Aat	
38%					



Mon. May 28th

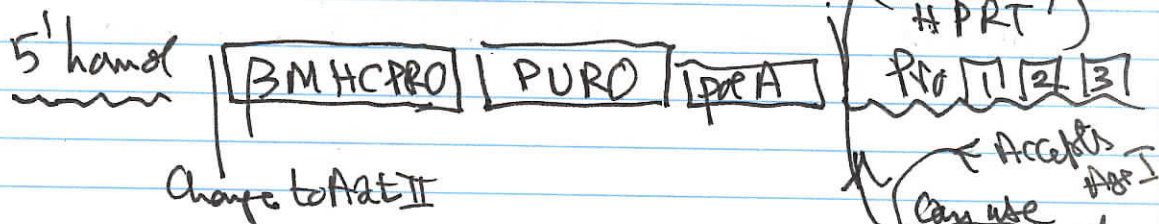
Planned hrGN.bMHC (promoter)



Planned ~~hrGN~~ α MHC (promoter)



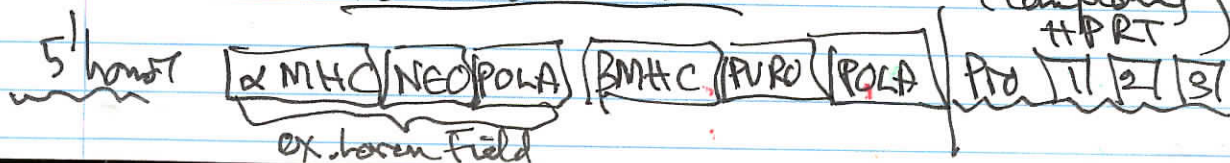
Planned bMHC PURO.POLA

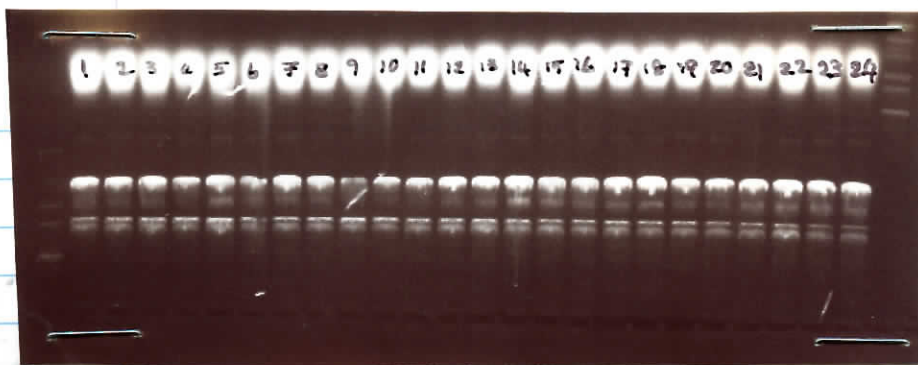


Planned α MHC NEO.POLA

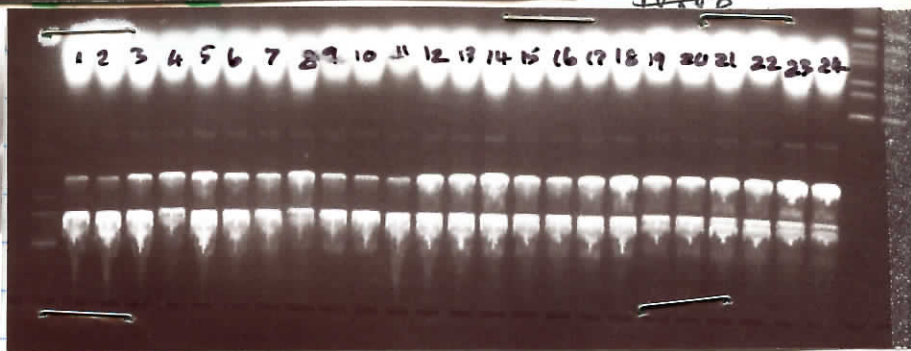
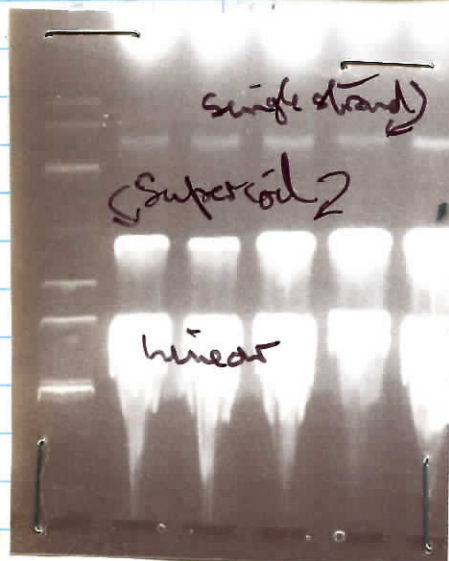


Planned double





① Sad day!
Not even one.
Not even Kpn cut.
Text original
planned to
redigest all
clones



② Not much better day. All cut to some degree
with Kpn. None cut with Spe.
Linear is >8 kb.

Conclusion: Try once more on longer ligature (L'53²) & if
not reconfirm the single cut frag^t (L'53) of GL3 β MHC PRO
Better to put effort into Hind & Age anyway.

1:25 pm
2:55 pm
0.8% HX4
max

Tues. May 29th

1 BMHC PRO
Spe
Hind

BMHC modification (contd. from L'53)

Agg W ①

Agg MM
① L'53
minis
Spe/Kpn

Test with Kpn & Spe I → expect 3.5 kb & 4.8 kb for correct. Incorrects will be more asymmetric.

② Agg MM W

1:42 pm
3:02 pm
0.8% HX4
max

Take 50 µl 10X R1 salts

✓ 442.5 µl ddw

✓ 2.5 µl (500) Kpn I lot 50 (200 µl)

✓ 5 µl (500) Spe I lot 28 (100 µl)

Agg W ②

20 µl aliquots + 1 µl L'53 minis ① - ② 37° 11:37 am to 1:07 pm

① L'53 minis
Kpn Spe
repeat

Wed. May 30th

Repeat digests

Kpn Mix
No NaCl

Spe Mix

Take 50 µl 10X NEB buffer 1 (pH 7.0)

✓ 50 µl 10X Alb.

✓ 395 µl ddw

✓ 50 µl (1000) Kpn I lot 50 (200 µl)

✓ 25 µl 10X NEB 3

✓ 25 µl 10X Alb

✓ 190 µl ddw

✓ 10 µl (1000)

✓ Spe I lot 28 (100 µl)

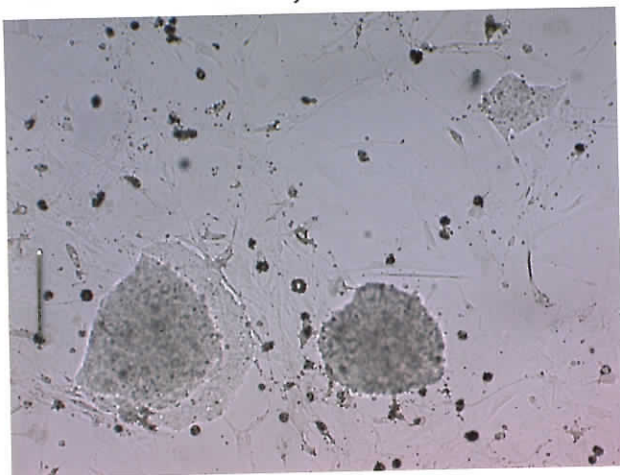
Blue 20 µl + 2 µl L'53 minis ① - ②

32° 10:35 am to 12 pm

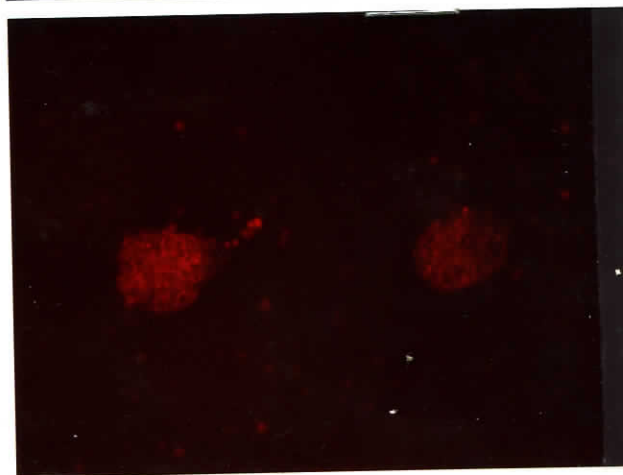
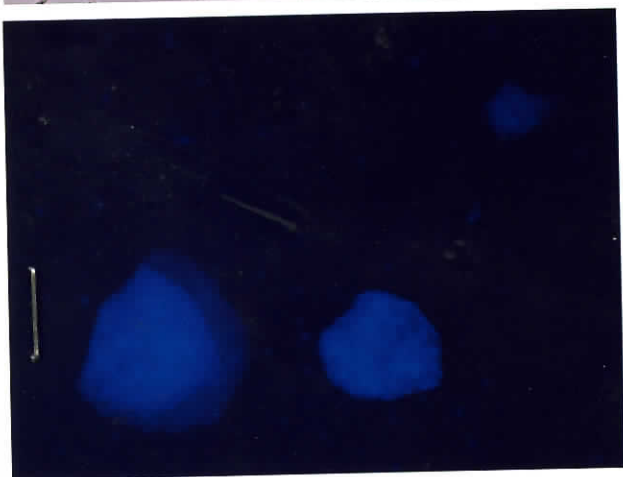
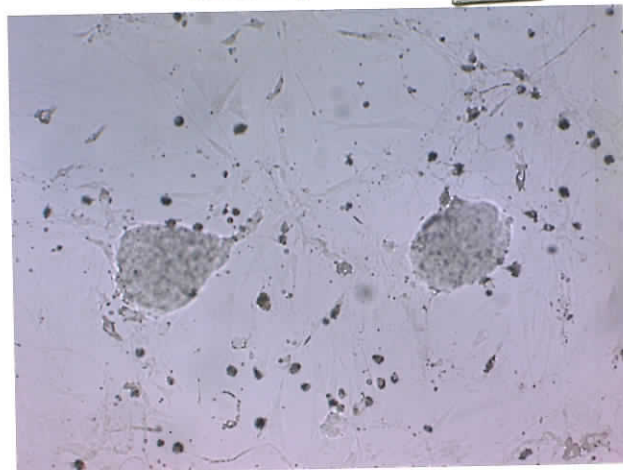
Red then 10 µl to 10 µl of buffer 3 (100 mM NaCl, pH 7.9) to give close to buffer 2 (50 mM NaCl, pH 7.9) containing 4 u Spe I 32° 12:12 pm to 1:22 pm

Rest 10 µl ddw add prior to running

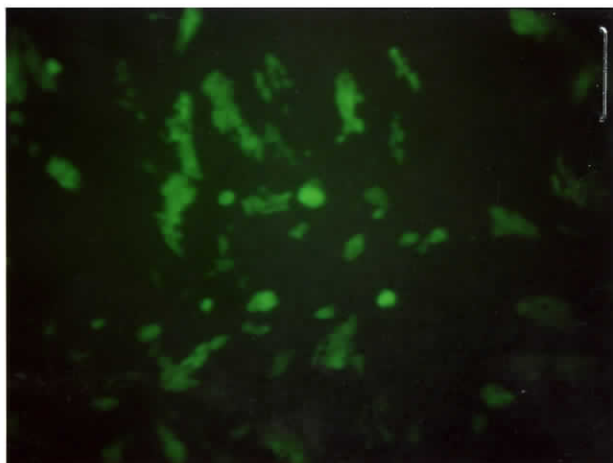
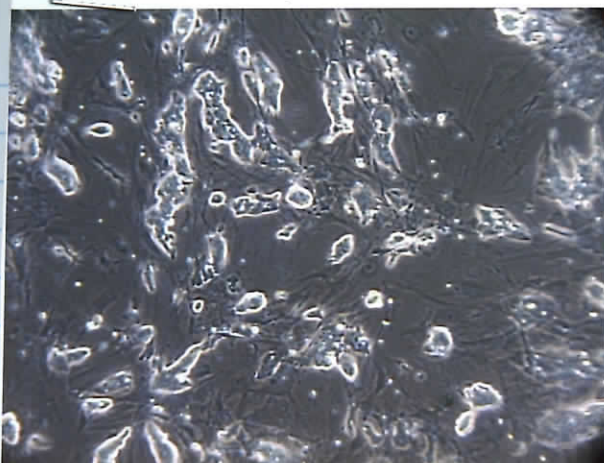
CCTA, in HAT



MRCTA, in HAT

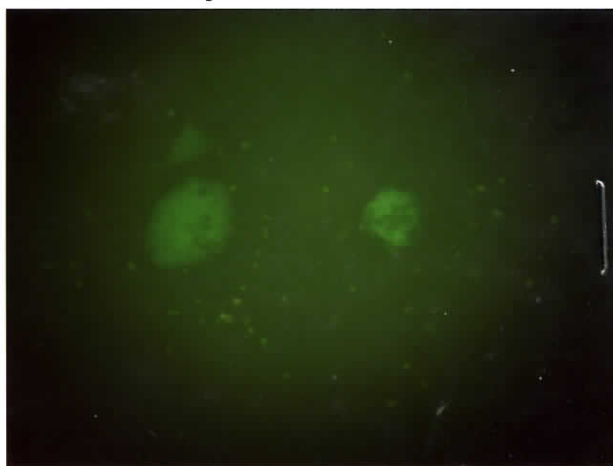
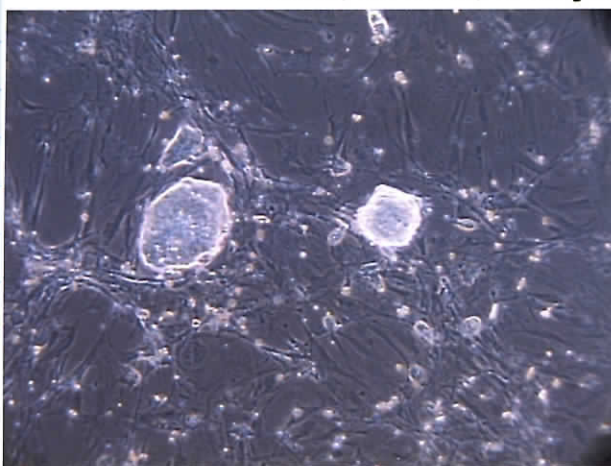


YCTA, +P10, derived from an individual colony



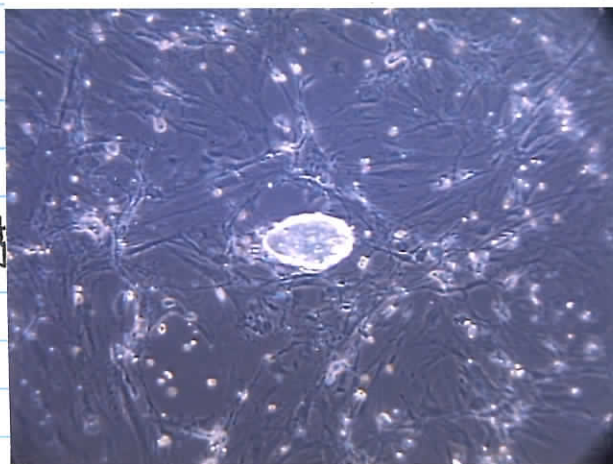
1/8 sec

CCTA, in HAT, 8 days after electroporation

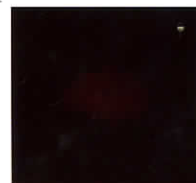


1/15 sec

M RCTA, in HAT, 8 days after electroporation



1/2 sec



1/8 sec

YCTA
looks
to
have
no
problems

CCTA
(some
filter)
also
looks
good,
but
needs
confirm.

M RCTA
has low
sensitivity
but
appears
okay.
Rad2
could
be
better.

Wed. May 30 ch

BMHC PRO Hunt → Age

65

Age

Hand

BMHC PRO

Ligase

✓ 10 pl ddw

✓ 2 pl L'53 Fragt

✓ 2 pl p95 10x ligⁿ buffer

✓ 4 pl L'34 Hind Age Hind stige

✓ 2 pl K'59 T4 ligase

add 5:05 pm to ~~3:30 pm~~ 3:30 pm Thurs to cold

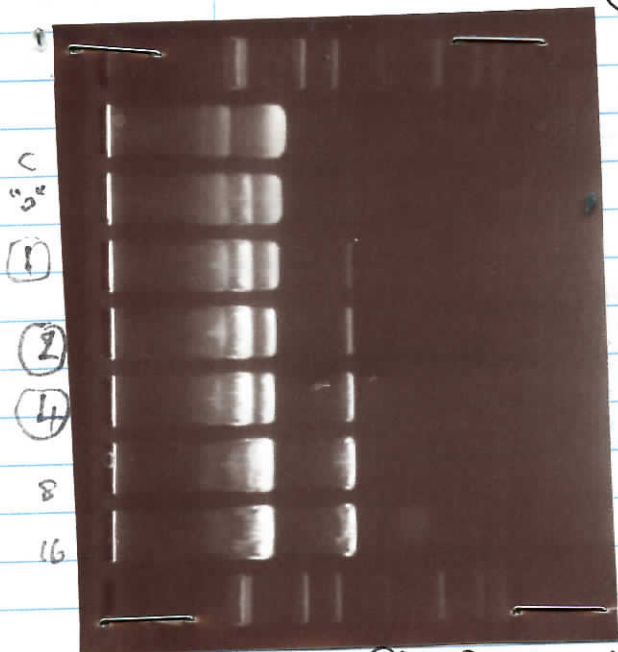
L'65

LIGⁿ

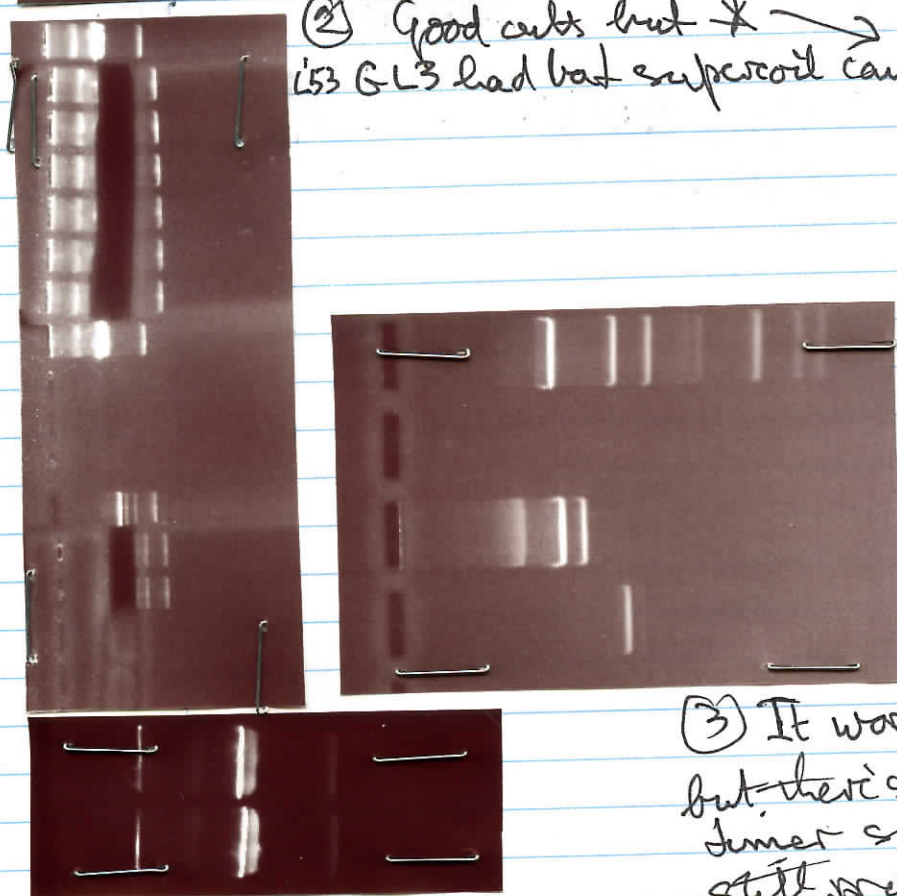
Transformation

3/12	3x5 d	80 pl ✓	1x AMP NZY	37° ~ 4 pm
	L'65 lig ⁿ	5 pl ✓	R. 20, 5, 20, 5	11
	0° 3:30 pm to ~ 3:50 pm		16, 16	11
	90 sec 42°			

L67
1, 2, 4
Pool



(2) Good cuts but * $\rightarrow$
153 GL3 had bad supercoil carbure.



③ It worked at 95° !

but there's a lot of
inner supercoil
still present. Proceed
with BMHC PRO. ? Tr

(No) and more purific - of 167,
using IV/am with redigested K147 w/5
(No) or proceed to screen many -

Byte
W
AG
C
"0"
1
2
4
8
16
AG
W
Byte

~~On 12:47 pm
 2011-1-32 pm
 2011-1-32 pm
 0.89 hr
 max~~

(3) Byt
W
Ag
W
L'67 hr GNss
5+10 L'67 G² frag⁺
W
ACR
W
Dye

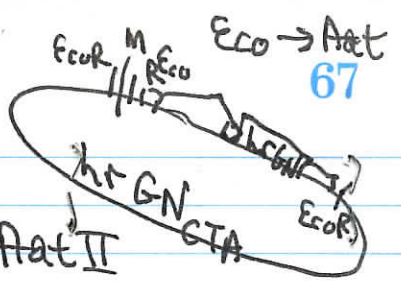
0.8% Hx4
max

byc
W
AG
C
"D"
1
2
4
8
16
AG
W
byc

①

Thurs. May 31st

hr GNCTA EcoRI → AatII



Single cut Eco

40 pe K'147 hr 5 (hr GNCTA) in 40 pe is rather too much for prep.

Take

✓ 20 pe K'147 hr 5 (hr GNCTA)

✓ 340 pe ddw

✓ 40 pe 10xRI salts

Warm to 37°

Add 1 pe (20) EcoRI hot 29 (200 pe)

✓ control "0", 1, 2, 4, 8, 16

60 pe " " " " " Rest 10 min at 80°

L'67
1, 2, 4
pool

47 pm
1:32 pm
0.8% Hx4
max

1.22
1.29

②

1V/cm No band

Decreased to 0.5 V/cm at 6:10 pm, 9:30 am

0.8% Hx4

Seal plaque

* Somewhat bad luck on CTA, the main band was still inside agarose?

Agarose gel

Cut & extracted but had to go to ~95° so may have lost sample.

L'53 frag⁺ cut was good & sample was clear from phenol but forgot CTA.

Proceed anyway with both. Wash GL3 with 25% EtOH. Run test gel on CTA.

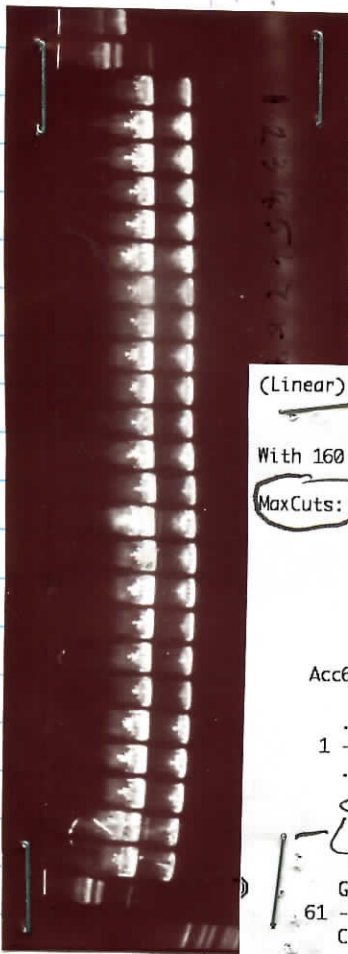
Wash frag

byc
W
3 slots
L'67
↓ 1, 2, 4 Pool
W
byc
W
↑ slots
3 L'53
↓ fragt
W
byc

L'67
hr GN ss
in 40 pe frag

L'67
GL3 ss
in 20 pe frag

L'67
7 W (5)

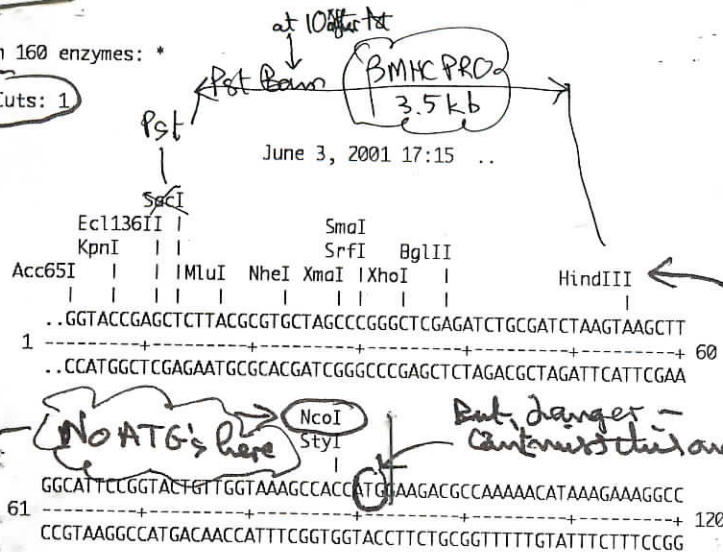


① Conclusion There are 2 Bam sites in construct
 - one at ~10 → a second (in GL3) at 2000 (+337)
 Since only two bands, there are no Age I sites
 in these constructs. Try again on return from
 U.K. using ~~BMHC PRO~~
 ? PCR to give At II & Age I ends. It would
 be 3.5 kb!! or just trying Hind to Age again.

(Linear) (MinSite=6) MAP of: pGL3.seq check: 4782 from: 1 to: 4820

With 160 enzymes: *

MaxCuts: 1



DNase 1: 0.8% Hx4 3/10 RNase

Dye ①

Pre L'73

Post L'73 Eco

① L'69 mini Bam + Age

Fri. June 1st

BMHC PRO Hind → Age (cald.)

Take 5 µl L'67 GLSS Fragt

✓ 7 µl ddw

✓ 2 µl 10x p95 lig. buffer

✓ 4 µl L'34 Hind Age Hind Eco

✓ 2 µl K'59 T4 ligase

24 AM L'69 lig.

15° 12:30 to 2:35, RT 2:35 to 4:38, 37° 4:38 to 5:15 pm

Transformation

1xAMP NZY 37° 6pm

3/12 DH5 α L'69 lig

80 µl

5 µl R, 20, 5, 29, 5

0° 5:18 to 5:42 pm

42° 90 sec.

Many ② ⑦ ~ 10 min to sort

Minis L'69 ① - ② 37° 5:40 pm 9:15 am Sat. Sun

Can use 2 µl in future here or above

Can excise with Kpn or Bam + test

BMHC PRO Age

Take 50 µl 10x RTI buffer

✓ 422.5 µl ddw

2.5 µl (50) Poam H1 lot 82 (20 µl)

25 µl (50) Age I lot 33 (2 µl)

Sun.

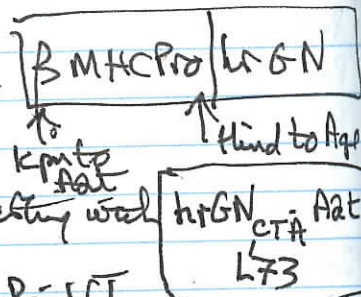
20 µl + 1 µl all minis L'69 37° 2 pm - 3:05 pm

Result shows shorter Bam site in GL3 at 2006
Eco cannot test well although I could excise.

No sign of Age site since all give ~ 6 kb + a shorter (presumably ~ 2 kb) but no band at 3.5 kb. (2 Bam + 1 Age would give 2, 2.8, 3.5; 2 Bam without Age w'd give 2.8-25.5)

Some notes

Can use these to make



AatII/Mlu or EcoRI/Mlu/AatII

AgeI

BstXI

EcoRI Extra HPRT Puro II

AatII

BMHC PRO

AgeI

BspEI

Need to know 9/F which is AatII Mlu

AatII

AgeI

Need to change Puro Bsm to BspEI.

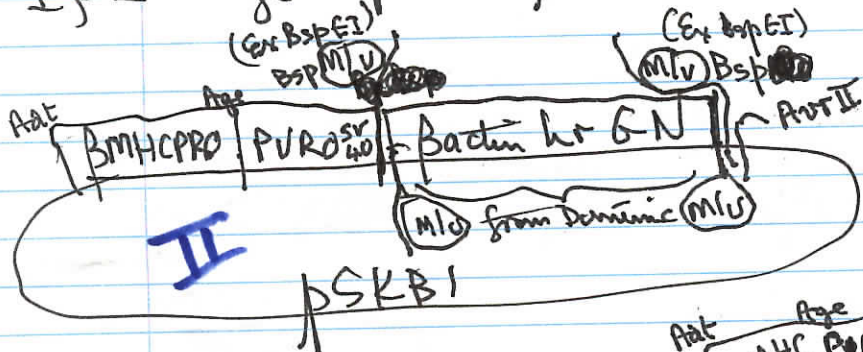
AatII

I

PSKB1

BspEI

Dominic is converting Mlu in hrgN to Aat to allow Mlu excision. If I change BspEI in BMHC PRO Puro to Mlu, I can make

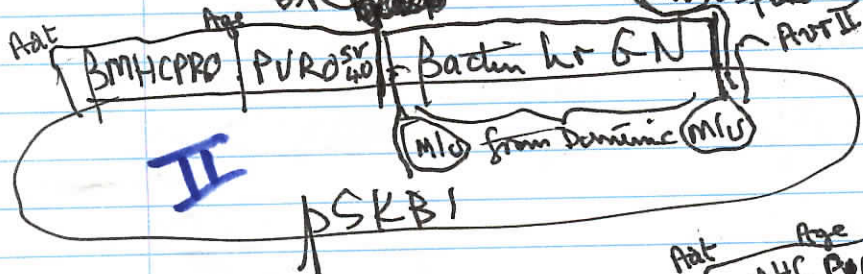


II

PSKB1

(Ex BspEI) BspEI

(Mlu) BspEI

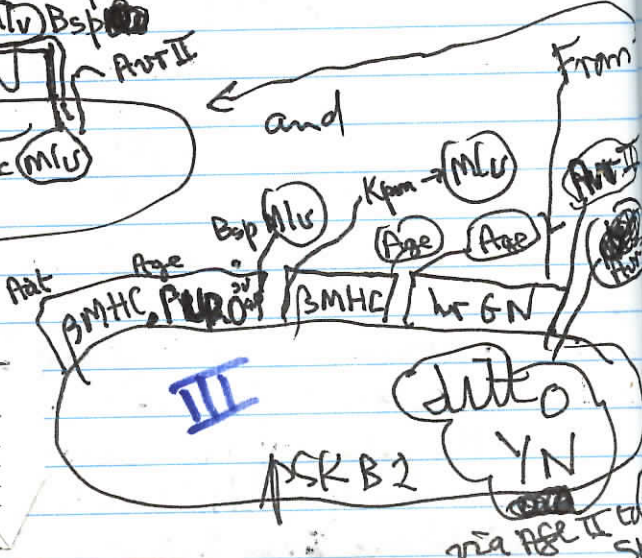


III

PSKB2

ditto YN

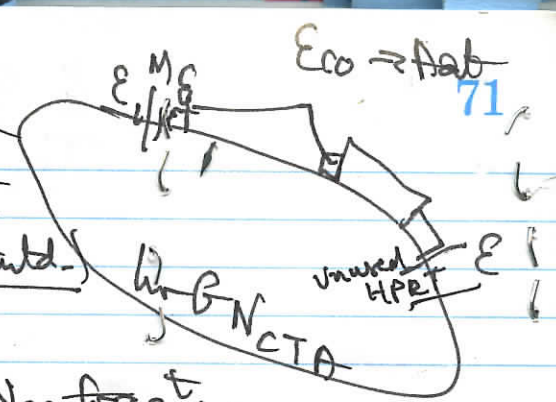
BamHI	L70	Bam	BspEI	Bam
GAT	CCT	GTC	CGG	ACA
G			BspEI	
16				
6390				



N
to Age
Aat

Fri. June 1st

hr GNCTA Eco → Aat (catal.)



L71
L6N

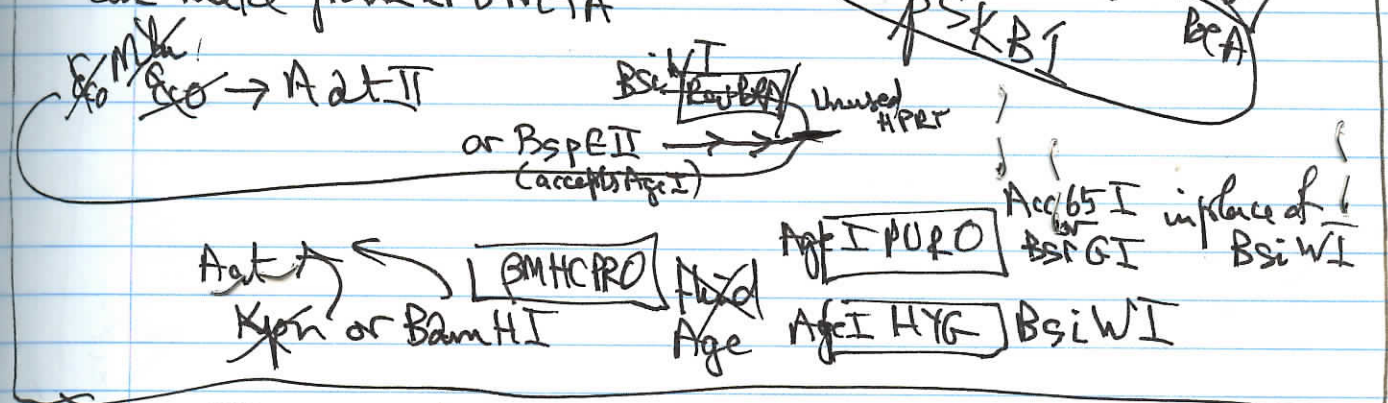
- ✓ 2 pl d.f.w
- ✓ Take + 10 pl L 67 hr GNCTA fragt.
- + 4 pl L 58 Eco Aat Eco lig
- ✓ + 2 pl p 95 10x lig. buffer
- ✓ + 2 pl T4 ligase K 59

Odd 2:30pm — 9pm Sat. → Cold

Sat. June 2nd

Planned

Can make from hr GNCTA



AatII → ~~PSKBI~~ AgeI Puro
KpnI or BamHI AgeI Hyg BsiWI

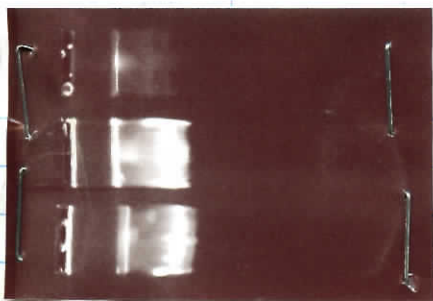
Transformation

ABANDONED

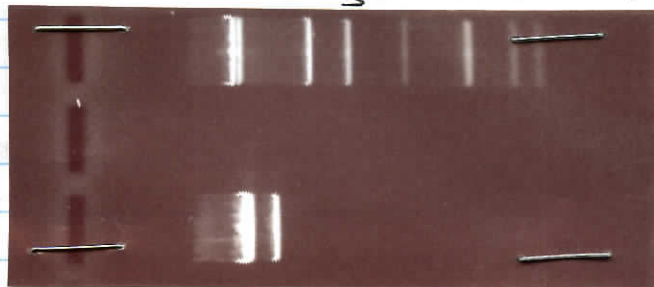
3/12 DH5α 80 pl Plate 1x AMP NZY
L71 lig 5 pl R, 20, 5, 20, 5
Sat 00 9pm to 9:25pm 16, 16, 16
90 sec. 420 37° 9:35pm (>40)

Too many colonies. Re digest → re purify.

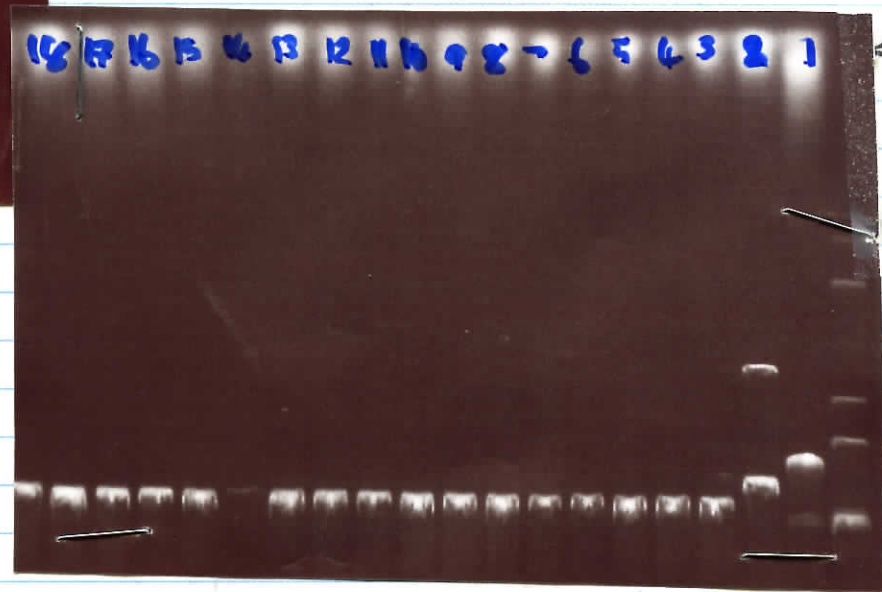
Sat. 1
Sun. 2pm



① Good cut but v. poor separation.
 Try using 0.8% Seap with 20%
 spike of 0.8% Agar to give
 a gel without an adhesive



② Fragment is, nevertheless, much improved.
 with much less time
 Proceed. (compare L'66)



←
 Aat II / Bsi WI
 digest
 →

Tamara ~~made~~ made 24 of L'73 - no positives. Then
 transformed longer ligata & picked 18 colonies. #1
 was positive

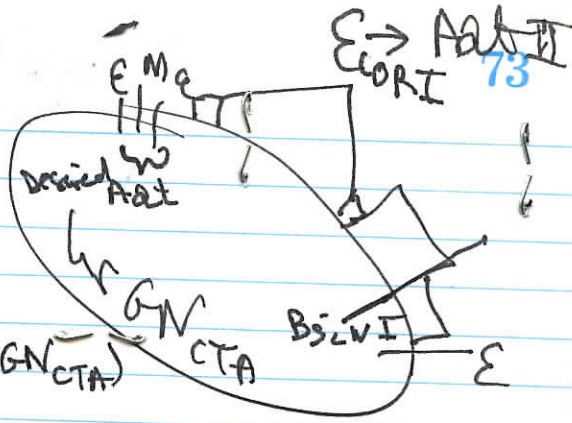
L'73
 hrGNetA

Sequence confirmed L'7b

1 pm
2:05 pm
0.8% HX4 after 10 min
MAX at 3 V/cm

Sun. June 3rd

Eco → Aat II (catd.)
hrGNCTA



123 hrGNCTA
W
Eye

Take 10 µl K147 hr5 (hrGNCTA)
170 µl Ldw
20 µl D+R1200
Warm to 37°
Add 1/2 µl EcoRI lot 29 (20 µl/µl)

L'73
Eco

15 µl - 37° 2 min. → to 380° for ≥ 10 min ✓
L'73
Prc

0.8%
Ag.
HX4
W
Eye
0.8%
Seap.
HX4
W
Eye
L'67 hrGNCTA ss frag.
L'38 hrGNCTA ss frag.
L'9 hrGNCTA ss frag.

Fragment back up into 75 µl as L'73 hrGNCTASS

ligation
Take 5 µl L'73 hrGNCTASS
4 µl L'58 Eco Aat Eco gligo
2 µl p95 10x lig. buffer
2 µl T4 ligase 459

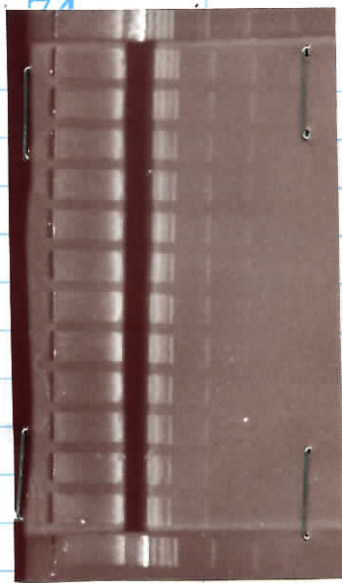
L'73
ligⁿ

Cold Transformation

3/12	DH5α	80 µl	1XNZY	1XAMP
L'73 lig ⁿ	5 µl	37° 6:55 pm		
00:52 pm to 6:46 pm	R, 20, 5, 20			
90 sec	4:20			
		(50) (40) (2) (2)		(0)

Digesting minis with Aat II and BsiWI will give a diagnostic band of 5 kb.

11 am #1's good distribution last.



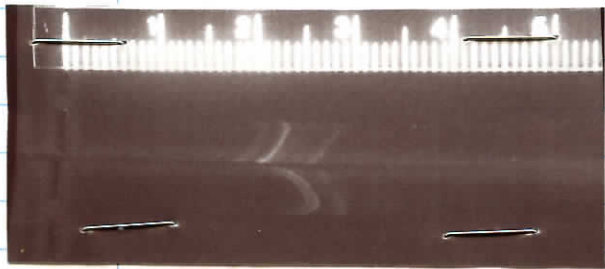
① Easy to handle. Solubelizes with phenol
at $\sim 85^\circ$. Separator fine. Cut good - but see
Recovered ~~as~~ as

L'75 Fragment
PM.HC PRO Hand
SS



② It was lost - the tiny bit left doesn't look v-good, but proceed.

③ Very odd. Don't understand this gel - Aban ton.



③

0.8% Hx4 max

W (2)

AG

W

L75 fragt (42yr)

W

ACR

W

L75
BMHC
H 33

2:35 pm
On 5:05 pm
3 V/cm

Mon. June 4th

(153) Repeat of Hind III single on BMHC PRO GL3

Take 35 µl 10x R1 buffer

309 µl ddw

6 µl (~bpg) L34 BMHC GL3

Warm to 37°

Add 0.6 µl (12u) Hind III lot 52 (200) µl

2 min. at 37° then to ≥80° for 20 min

Hind → Age 75

BMHC PRO
Single cut Hind

①

14 slots

Hx4

0.8% Seap 40

0.8% Ag 10

Washes well with blotting cloth.

Hold at CIAA

* Prob lost ppt. from 400 µl → into 4.2 µl
Recovered from 80 µl → into 8 µl

Take 5 µl L75 Fragment BMHC PRO Hind SS (8 µl)

4 µl L34 Hind Age Hind oligo

7 µl ddw

2 µl 10x p95 ligase buffer

2 µl K59 T4 ligase

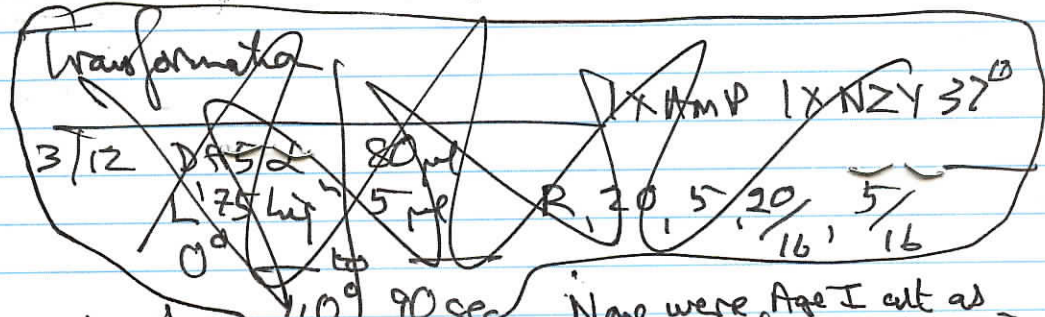
L75
Hind
BMHC PRO

15:10:12 am

RT 12:50 pm

37° 1:50 pm

N 2:20 pm



To Tanaka for transformation
Mon. June 11th

None were Age I cut as judged by Tanaka II/Age I - sh'd give 3 bands in place of 2.

Repeated the ① gel (3) On 3:33 pm

Hx4 Agarose 20
0.8% Seaplaque 80

Tanaka will try more columns

Cancellation

was made because the partial Eco was successful from L'73. Check with: —

OK

175.seq

L'73 hrGN^{CTA}. Aat
with K'17 Blue Mlu primer

L'76

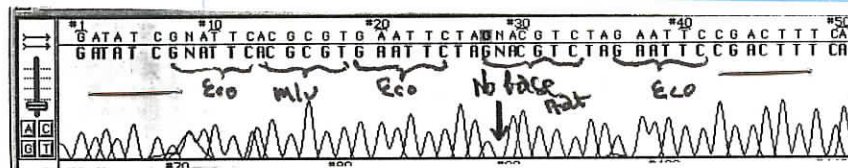
PSKB1TESTCODE.REV POLA

~0.2 pg/μl cut with Ago I & BsiWI
from kin

This is identical to CODETEST except
that the test sequence & its
promoter etc. are oriented
outwards from HPRT promoter

Sequence confirms correct modification.
hrGNeta. Aat II is therefore

Eco Mlu Aat II Eco Eco
| | | | |
fact



175.seq check: 5807 from: 1 to: 874
/afs/isis.unc.edu/home/n/o/nobuyo/oliver/hrGNeta.seq check: 1233 from: 4472
o: 19262

ASSEMBLE May 7, 2001 13:14

Gaps: 7 Quality: 7810 Ratio: 8.998 Score: 767 Width: 28 Limits: +/-29

EcoRI MluI EcoRI Aat II EcoRI

1 GATATCGAATTCACGGCTGAATTCTAGACGCTAGAAATTCGACTTTCA 50

||||| |||||||

4489 GATATCGAATTCACGGCT.....GAATTCGACTTTCA 4521

51 AACAGCCTTTGGACTCAGGCCTTGGGGACATTCAGGGGACCTTTACCA 100

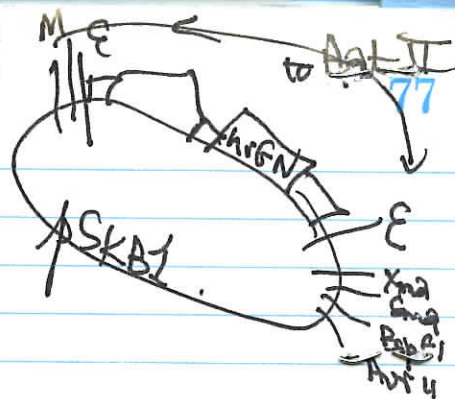
|||||

4522 AACAGCCTTTGGACTCAGGCCTTGGGGACATTCAGGGGACCTTTACCA 4571

Tue. June 12

New start for pSKB1.Aat

Cancelled



- Take
- ✓ 2 μ l 10x RI salts
 - ✓ 1 μ l K147 hr 5 (hr G-NCTA)
 - ✓ 16 μ l ddw
 - ✓ 1 μ l (20) EcoRT Lot-29 (200 μ l) 1/10

L177
pSKB1(Eco)

diluted to 20 μ l in 1x RI salts

37° 9^{am} to 12:45 pm

- Take
- ✓ 8 μ l 10x RI
 - ✓ 8 μ l ddw
 - ✓ 1 μ l (20) Eco RI

heat inactivated 80-85° ~ 10 min

Rationale: digest to completion. Re-ligate $\rightarrow$ pSKB1.Eco
+ oligo $\rightarrow$ pSKB1.Aat

Can then use the XbaI-Aat II sites for 3' end & Eco or Aat for 5' end. Spare sites will be MluI, NotI, Pml I & others (Age & Bgl)

Can then assemble from 5' to 3' ends in 3 or 4 fragments.

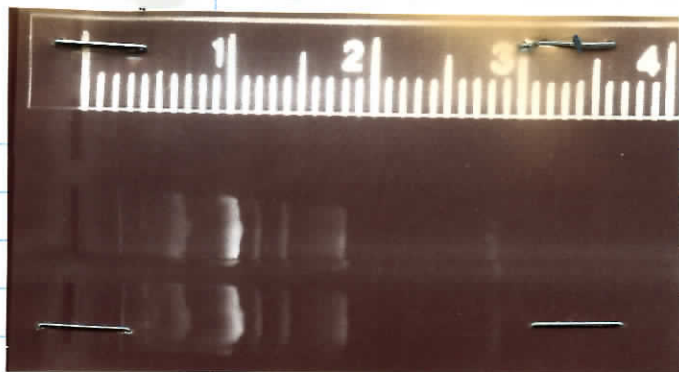
Take L177 Blue Eco (Take L177 Red Aat)

- | | |
|----------------------------------|------------|
| 1 μ l L177p SKB1(Eco) | 2 μ l |
| 2 μ l 10x p95 lig. buffer | 2 μ l |
| 15 μ l ddw | 10 μ l |
| 0 μ l L158 Eco Aat Eco oligo | 4 μ l |
| 2 μ l T4 ligase | 2 μ l |

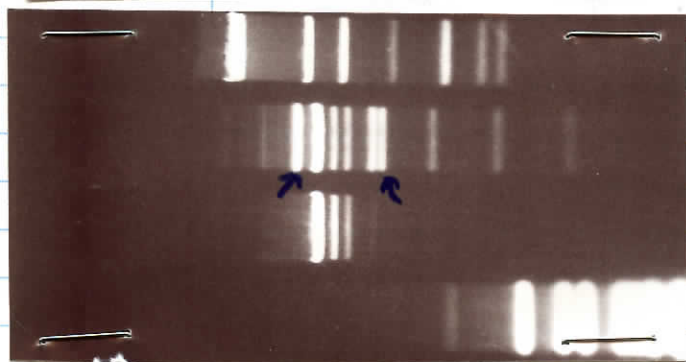
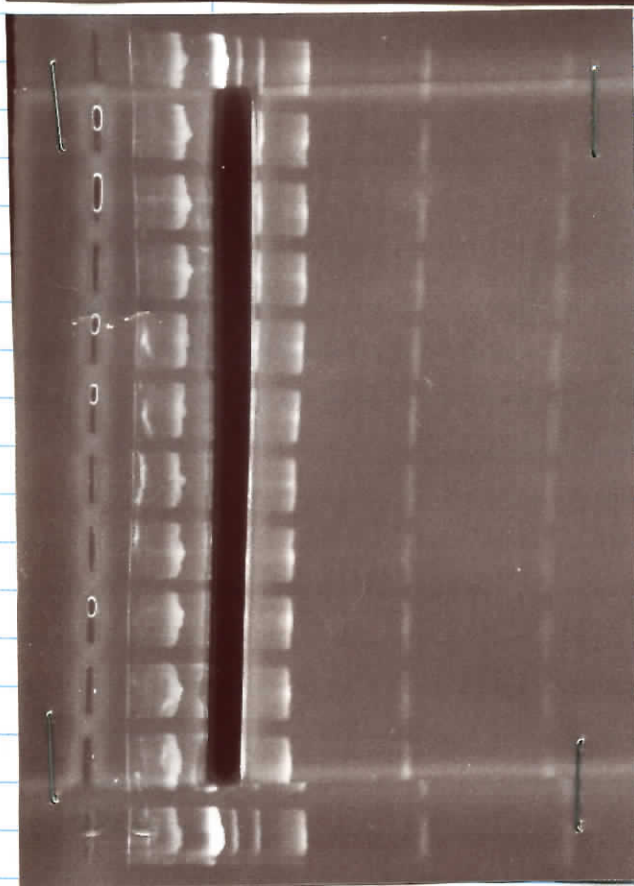
15° to RT to 37° to

Transformation

Blue Eco	Red Aat
L177	L177
<u>CANCELLED</u>	



① Digestion is optimal.
Good separation $\rightarrow$ cut (9mm/12mm)



② Nice frag^t. Recovery OK.

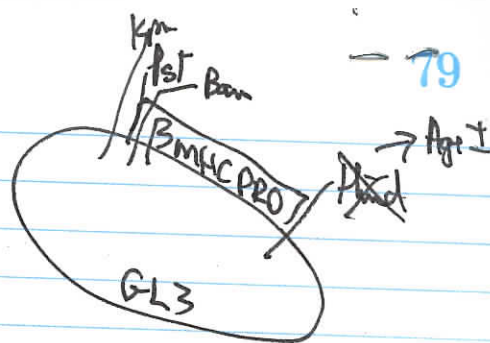
$\nearrow$ $\nwarrow$ show supercoil $\rightarrow$
supercoil dimer. See L'52.
Frag^t is virtually lacking
these two.

②

9mm/12mm)

Tues. June 12th

Single out β MHC PRO (L'34)
again again



L'79
BMHC
PRO GL3
ss Hind

✓ Take 35 μ l 10x RT buffer
✓ 309 μ l ddw

✓ 6 μ l (v bpg) L'34 BMHC GL3

✓ Warm to 37° (8 min.)

✓ Add 0.6 μ l (12v) Hind III L'34 52 (200 μ l)

Tomoko will make more.

✓ 2 min at 37° then to $\geq 80^\circ$ for ≥ 20 min ✓

①

0.8% Dye Agar Hx4 slots
0.8% Soap Hx4
ON 4 pm 8:50 am
OFF 1 V/or
L'79 BMHC PRO GL3 ss Hind
Recovered into 50 μ l as

1st blue just going off.

2nd blue $\sim \frac{1}{2}$ way

L'79 BMHC
Frag: β ssH

On 11:04 am
Off 11:54 am

②

Dye
Ag
L'79 BMHC PRO GL3 ss Hind
L'79 Frag: β MHC ssH
ACR
W
Dye

Ligation

Take ✓ 5 μ l L'79 Frag to
✓ 4 μ l L'34 Hind Age
✓ 7 μ l ddw
✓ 2 μ l p95 10x lig? buffer
✓ 2 μ l K'59 T4 ligase

15° 11:13 to 2:13 pm
RT 2:13 to 3:13 pm
37° 3:13 to 3:53 pm $\rightarrow$ Cold

Transformation

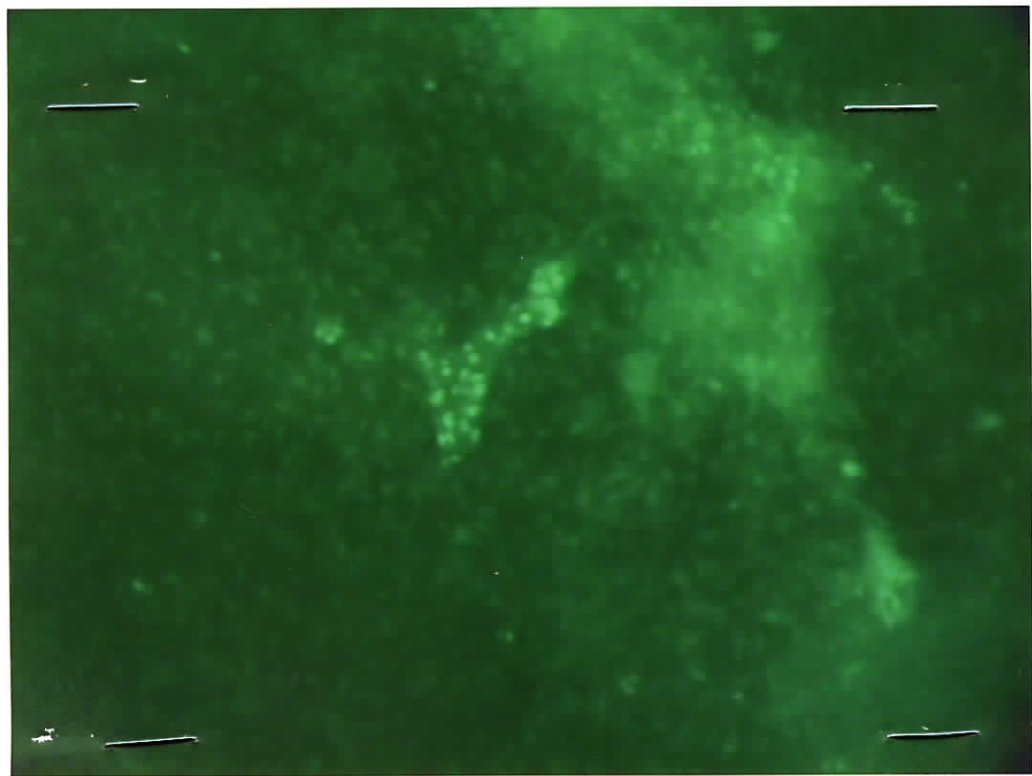
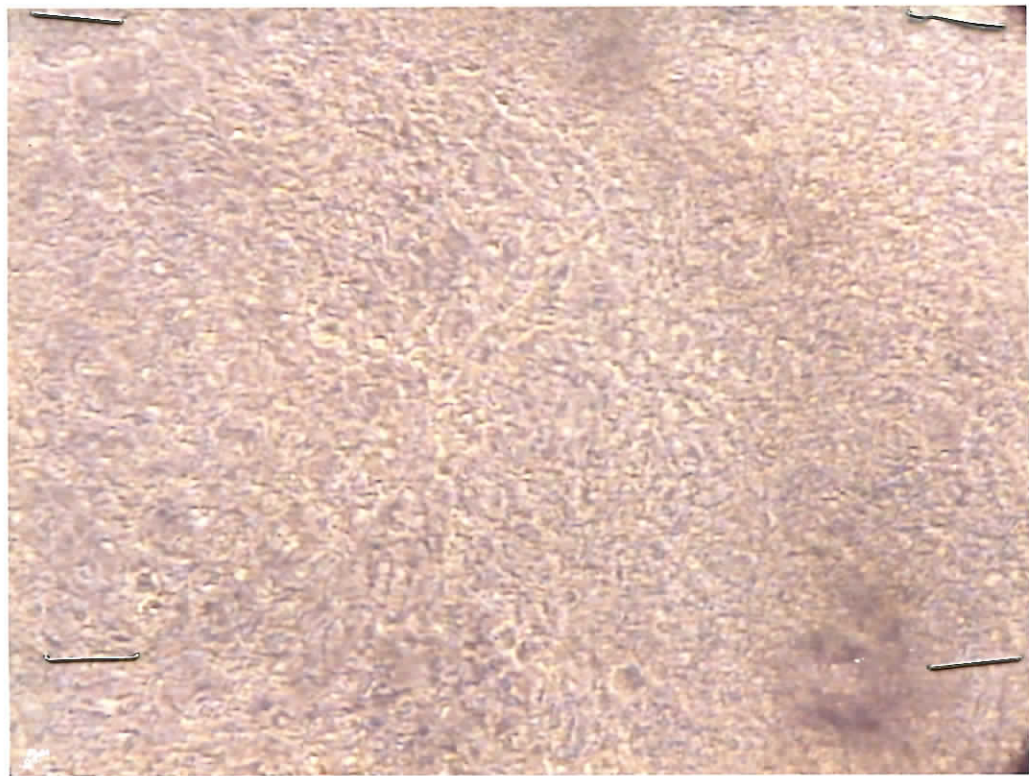
DH5 α 80 μ l ✓
L'79 lig 5 μ l ✓
On 3:54 to 4:34
90 sec. 42° ✓

1x AMP NZY

Plated many (230) (8) (3) (0)

37° 4:45 pm

Tomoko will make minis & test.



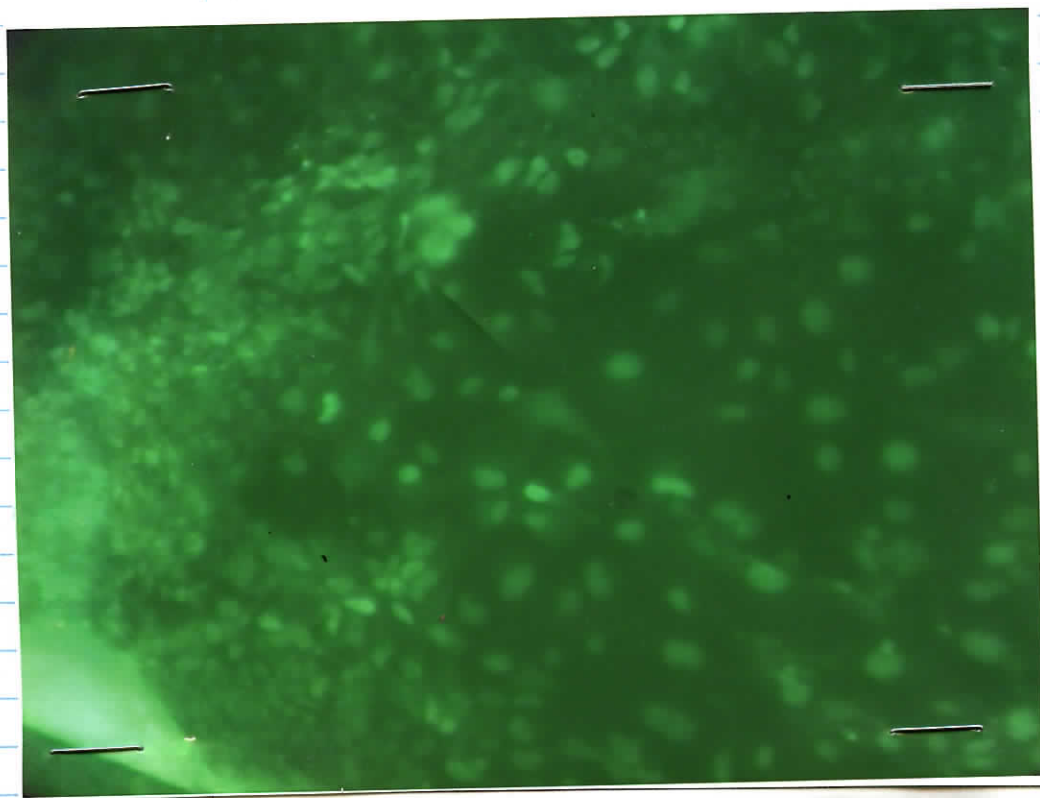
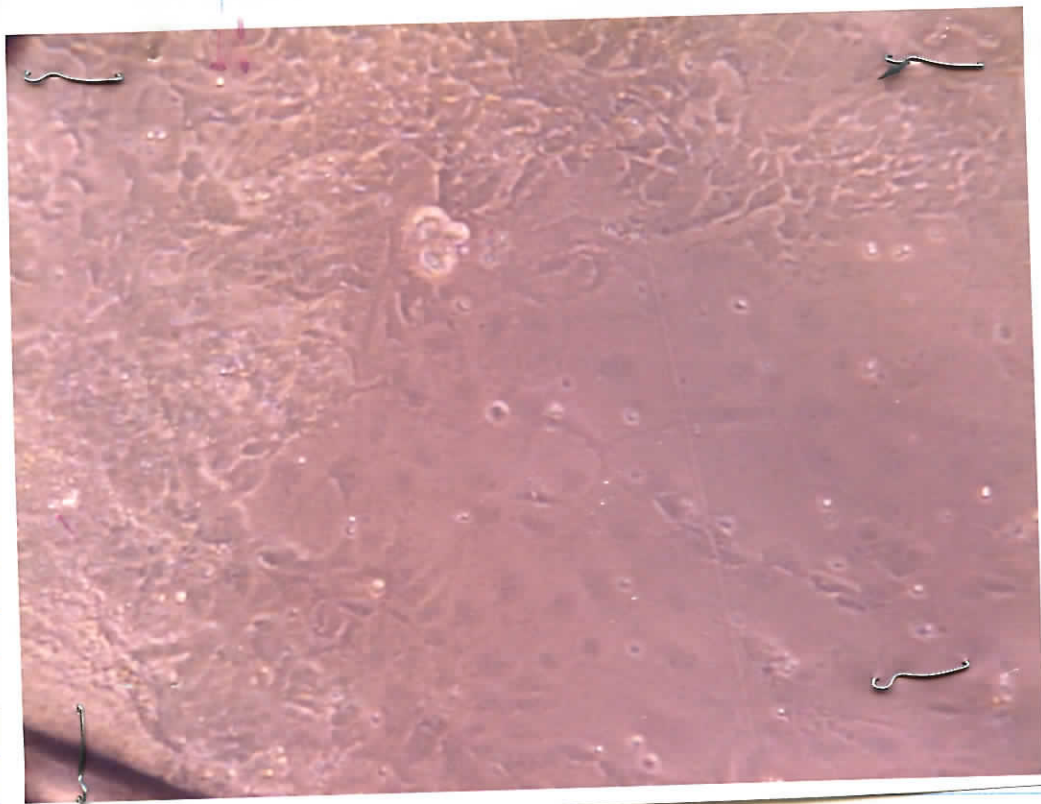
Tues. June 12th

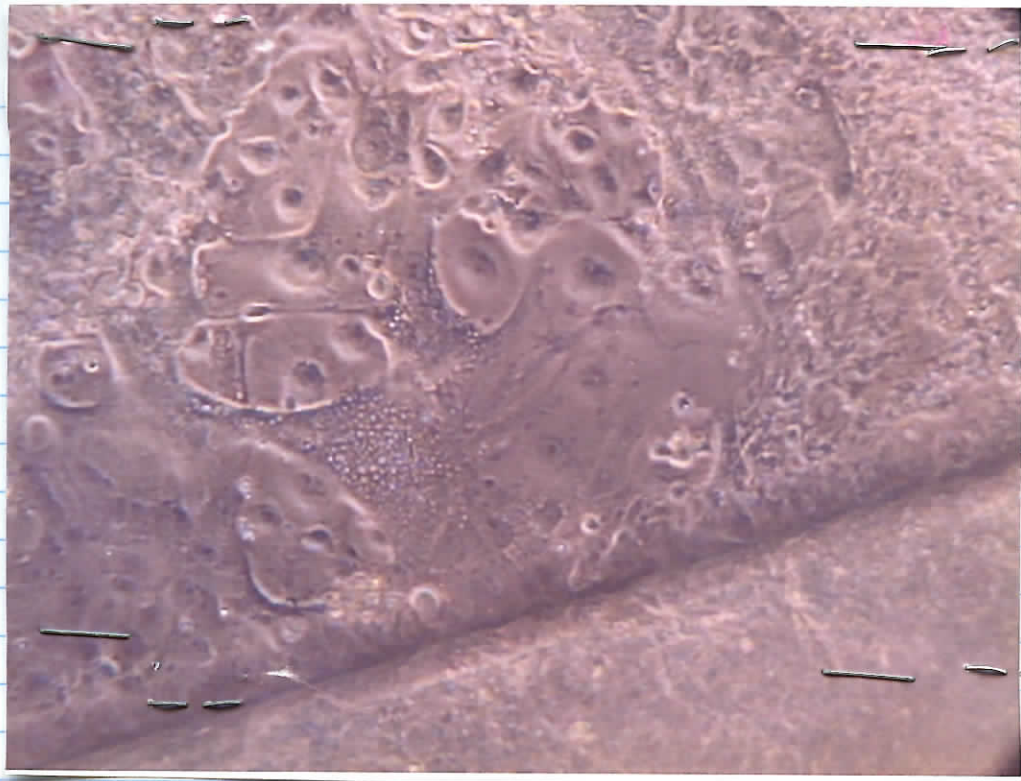
81

Same hr GN / cardiomyocyte results

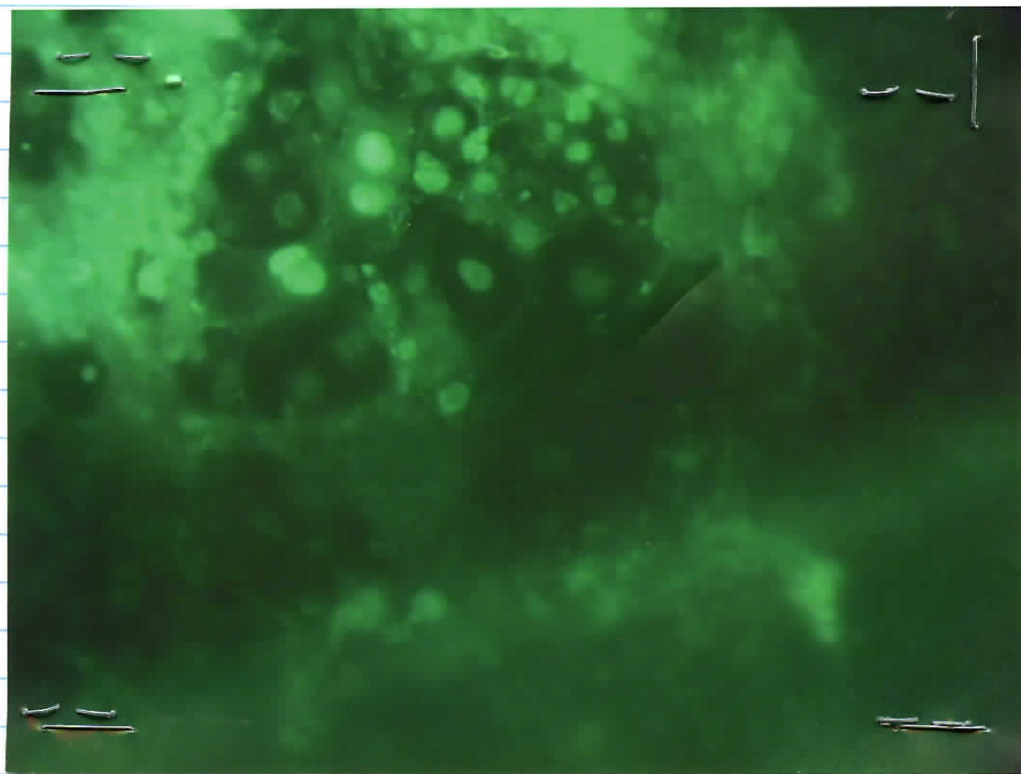
Joc Portchham from Wayne Cassio's lab has been following cultures of cloned ES cells with hr GN in the APRT locus using pSKBI CODETEST. REV/POLA. Nuclear fluorescence is v. cell-type dependent, but is beautifully strong in beating (video records) myocytes. Proceed to Lott CN & YN

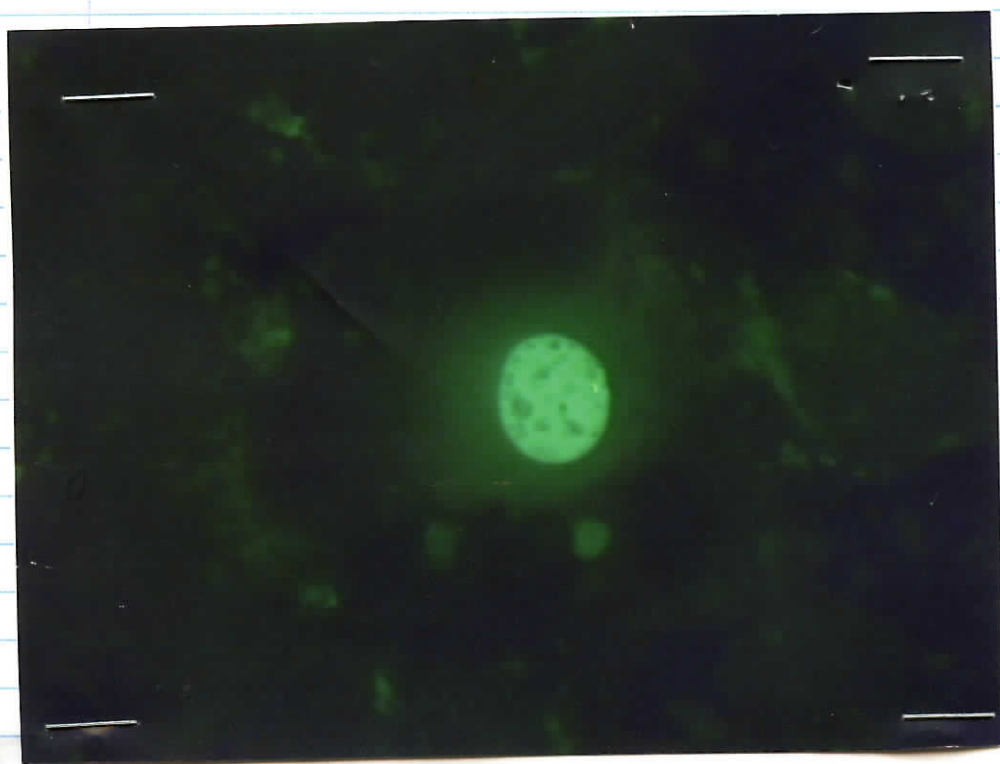
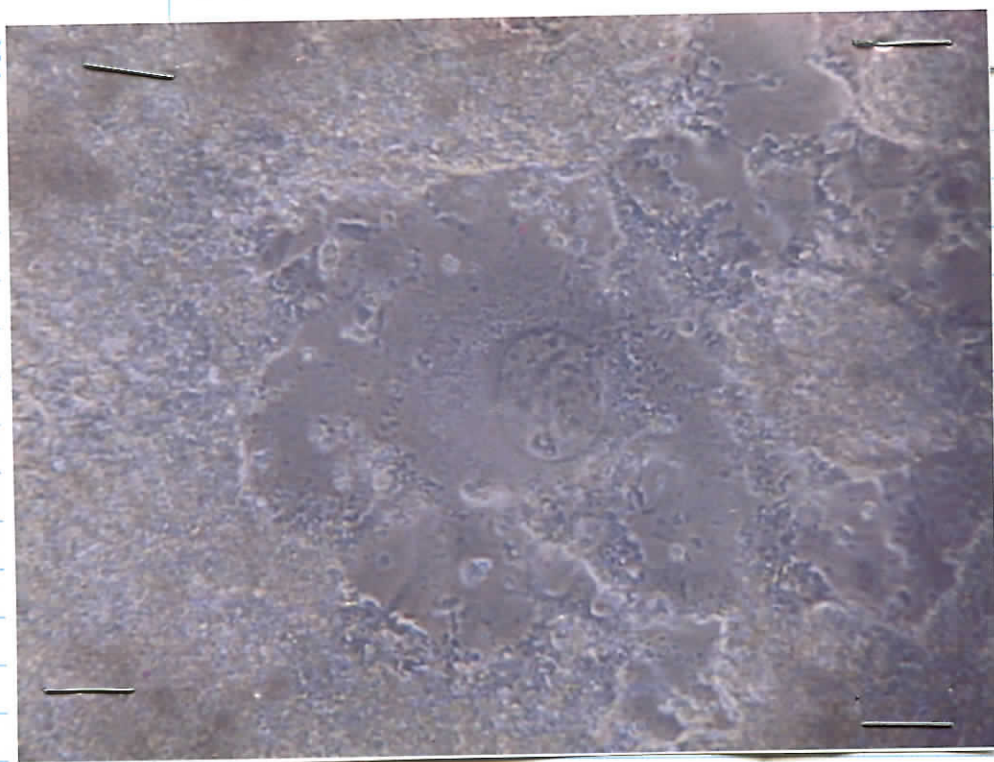
The cardiomyocytes are clearly binucleate i.e. $\equiv$ post 14 days
- the bright cells were beating!

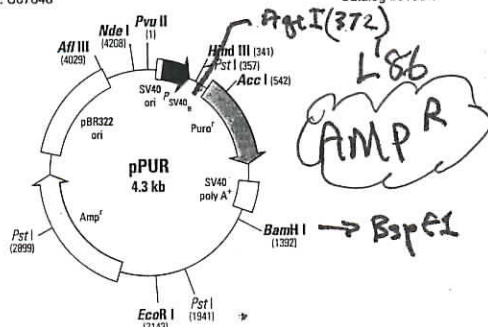




83

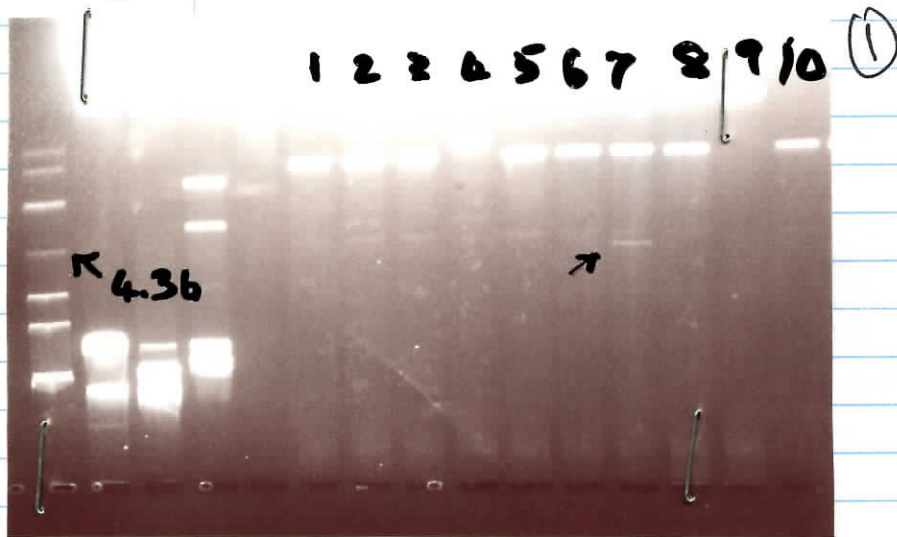






① Since plasmid is 4.3 kb, none are linear 4.3 kb, but I don't know why the plasmid is so contaminated with 2 shorter forms. But

check again with better enzyme. #7 may have a minute amount of cut.



L87 ~~Pre~~ | Gel on L90 Conclusion
L87 Bsp
② #7 is not any good.

01:52 2:22
 0.86 H4
 AGMM
 L91 PR5
 L91 Post Act

[Step (iii)]

Thurs. June 14th

Age
 PURO
 BspEI
 87

L91 Plat/Bsp (Quickie!) + PUR Bam HI to BspEI
 1392

L87 mini
 BspEI

L87
 PPUR
 Bam

Take 5 μ l 10x RI salts
 40 μ l ddw
 4 μ l L86 + PUR (2 μ l)
 0.5 μ l (0.40) Bam HI hot 82 (20 u/ μ l)
 37° 1:40^{pm} to 2:50^{pm} inactivated $\geq 75^\circ \geq 10$ min

This BspEI site
 not be Bam me.
 sensitive

my old
 data. 5' at 60
 not
 recombin
 20 min at 80°

ligase Take 5 μ l (0.2 μ g) + PUR Bam
 2 μ l 10x p95 lig. buffer
 7 μ l ddw
 4 μ l L70 Bam BspEI Bam oligo
 2 μ l T4 ligase K59
 15° 3:05^{pm} to 4:24^{pm} RT 4:24 to 4:56 37° 4:57 to 5:36^{pm}

L87
 L6

Fri
 Plates to Tomoko / Seago
 for minis + BspEI digestion
 They set up. I processed.

Transformation

L87 minis (1) - (24) Salt

DH5X
 L87 lig.
 0° 5:37^{pm}
 90 sec.
 looking good
 80 μ l 10x AMP P1KNZY
 5 μ l (20) (6) (0) (0)
 5:58^{pm} R 20, 5, 20, 5
 42.5°
 37° 6:10^{pm}

Take 50 μ l 10x RI salts

445 μ l ddw

5 μ l (50 u) BspEI hot 2 (10 u/ μ l) (1992)

8-20 μ l aliquots + 1 μ l minis 37° 11^{am} to 11:30^{pm}

Take 14 μ l 10x NEB buffer #3

34 μ l ddw

2 μ l L87 mini #7

1 μ l (100) BspEI hot 3 (1996) bottom

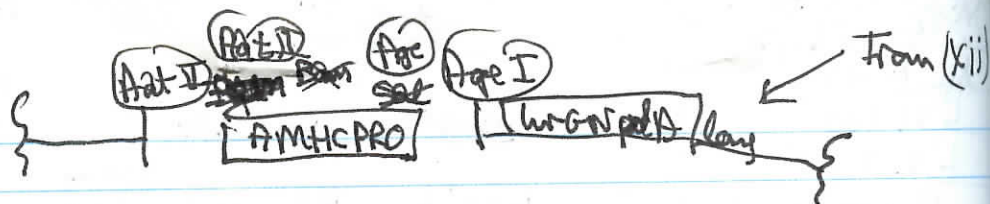
L87
 Bsp

18 μ l
 L87
 Pre

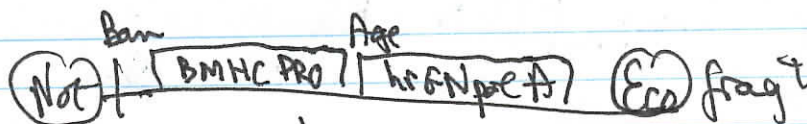
37° 9^{pm} to ~ midnight
 of tube 100/ μ l

+ 5 μ l (bottom)
 hot 3 (1996)

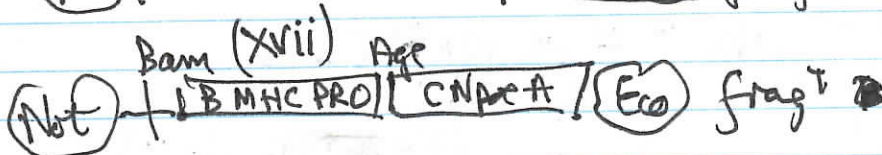
(xix)



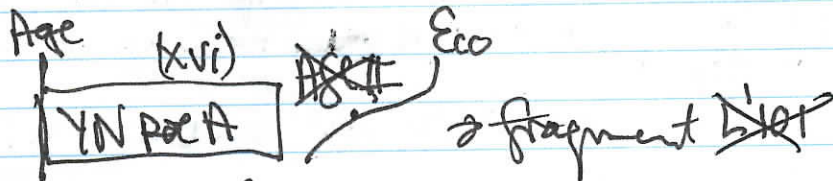
L'139 (xiii)



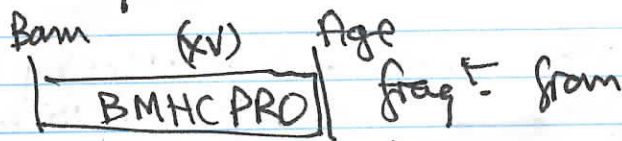
L'143 (xvii)



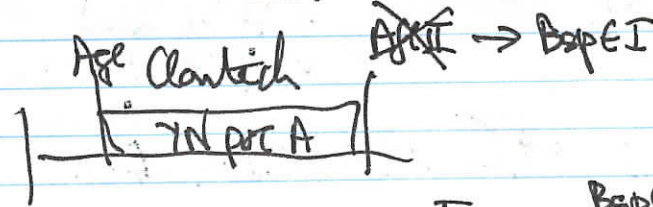
L'142 (xvi) M'7



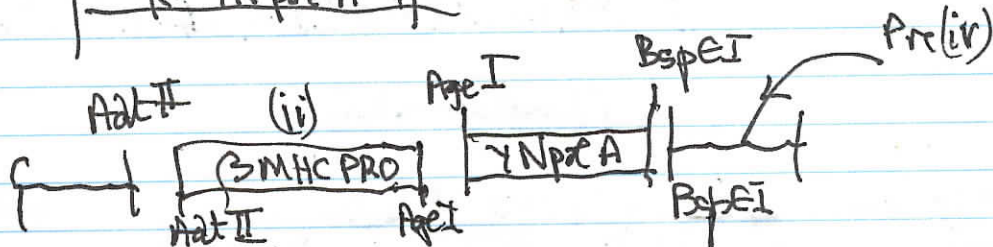
L'143 (xv)



(xiv)



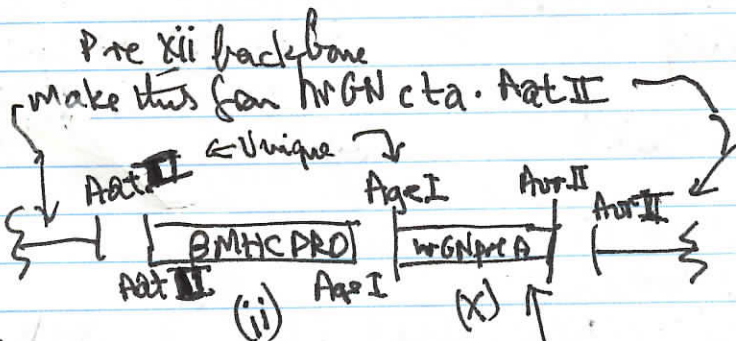
(xiii)



L'119

Useful (xii)

scan
w/ later
so BMHC w/ GN preparation.



Simple at

Can shorten this with Not I just 3' to pA

↓
BspEI
Cut → religate

Orig	29180	Afe II	Spe I
Date made			
Length	16		
% GC	56%		
	CTTAAGGCAC TAG TGC		
	CTTAAAGGCAC TAG TGC		
	CTTAAAGGCAC TAG TGC		
	CTTAAAGGCAC TAG TGC		

from (xii)

Page with
Completion

Fr. June 15th

More thoughts (see L'70)

Unordered
Steps:

✓ L'101

i)

BMHC PRO

Hind^{III} → Age I

Mlu^I → Age

or BMHC PRO

L'109 L'103

ii)

BMHC PRO

[from (i)]

clone ✓
fragment

L'97

iii)

Age (from Clontech)
PURO. SV40

Bam^I → Bsp^{EI}
[from Clontech]

L'107

iv)

Aat^{II}

Aat^{II} (ii)

Age I

(iii)

Bsp^{EI}

Aat^{II}

Useful

L'103 Backbone from hr GNcta. Aat^{II}

BMHC PRO

PURO. SV40

Active
Fragment

v)

(iv) BMHC PRO PURO
SV40

Bsp^{EI} → Mlu

Aat^{II}

from BMHC PRO PURO (iv)

L'113 L'105

vi)

BMHC PRO

[from (i)]

(From hr GNcta)

V. useful

vii)

(v) Mlu^I
BMHC PRO PURO
SV40

(vi) Age I

(v)

Aat^{II}

Mlu^I

Age I

Aat^{II}

clone ✓
fragment

L'95

viii)

Age
VNpA

Aat^{II} → Spe^I

[from Clontech]

V.V. useful
(cf. step VII)

ix)

(iv) Mlu^I
BMHC PRO PURO
SV40

(vi) Age

viii)

Aat^{II}

Age I

Mlu^I

Aat^{II}

Age

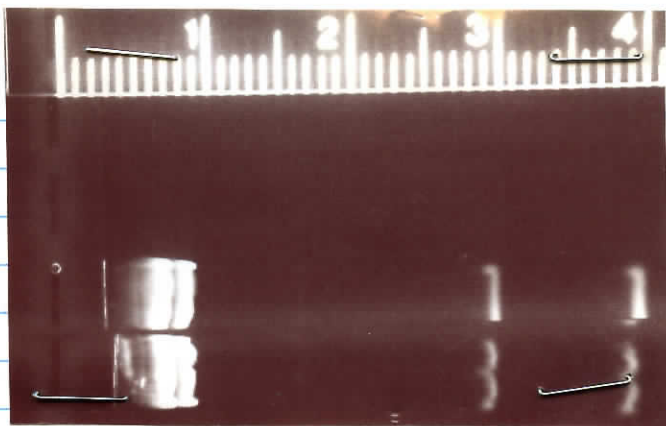
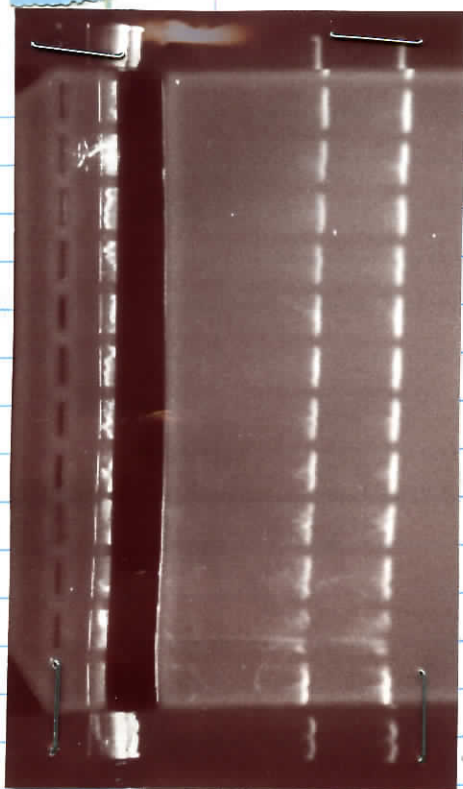
Spe^I

L'109 L'109

(x)

hr GN (SV40)

from hr GNcta



① Clearly the Aat II was incomplete.
 Tried for L'91 Fast ^{Blue} = Complete?
 → L'91 Slow ^{Red} ← incomplete?
 Test these again with Aat → Bsp.

↑ Below
 marker
 notes

[Step IV]

best in cold for
 ~10 bands

Catch-up
 gel on

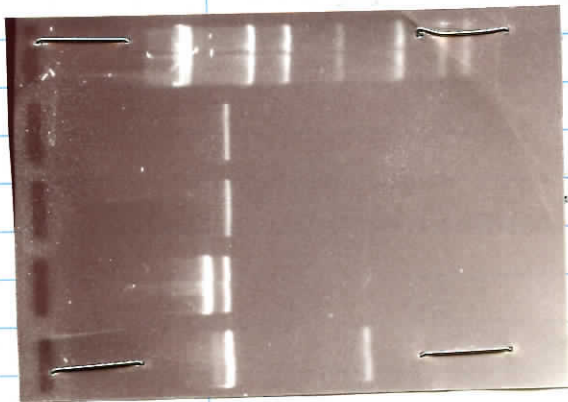
3:30 PM
 4:40 PM
 0.8
 1.0



②

③
 AG MM
 L'91 F Aat
 L'91 F Pae
 L'91 S Pae
 L'91 S Aat
 L'87 (mini)
 L'87 (mini)
 L'95 Pae
 L'95 Aat II
 ACR MM
 Water
 Dye

③ Diagnosis was correct (colias)
 L'91 Fast is correct for step IV
 but yield was less.
 Can remake when needed,
 with new batches of Aat II
 → Bsp EI, Repeated
 on L'103.



Dye
 Water
 L'99
 L'99
 128
 Dye
 68
 Ag
 84

Diagram illustrating the structure of the *hGNCta* gene, showing the *Pst*II site, the *Bsp*FI site, and the *Pst*I site. The gene is flanked by *Pst*II and *Bsp*FI sites, and the *Pst*I site is located within the gene.

10/24/27

M
Aat
F Pre
S Pre
S Aat
(χ^2 in F) Pre
(χ^2 in F) Bsp
S Pre (Yn)
Age II (Yn)
M M
er
e

Diagram illustrating the structure of a 4x4 grid with handwritten labels and annotations:

- Top-left cell: 0.89
- Top-right cell: 0.89
- Second row, first column: w
- Second row, second column: w
- Third row, first column: w
- Third row, second column: w
- Bottom-left cell: 0.89
- Bottom-right cell: 0.89
- Annotations: "4x4" is written on the left side, and "4x4" is written on the right side. A bracket indicates the width of the grid.

2nd 1/2

Sunday

16 yr

Take 6 yr L'91 Fast

✓✓ 3, 2 yr NEB 10 x 4

✓✓ 23 peddw

✓ 2 yr (8u) ART II lot 33 (1992)

370 1:45 pm

ditto with L'91 slow ✓

L'91
 S Aat

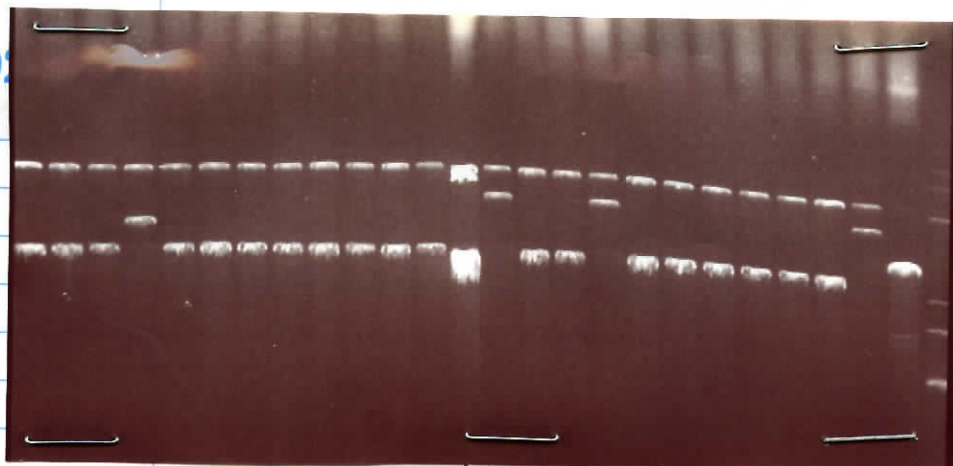
L'91 S Pre

Continued on L'103

Continued on L'103

On 10/11/12 5:00 pm Fri Off 11am Sat 2nd blue 43 min

9

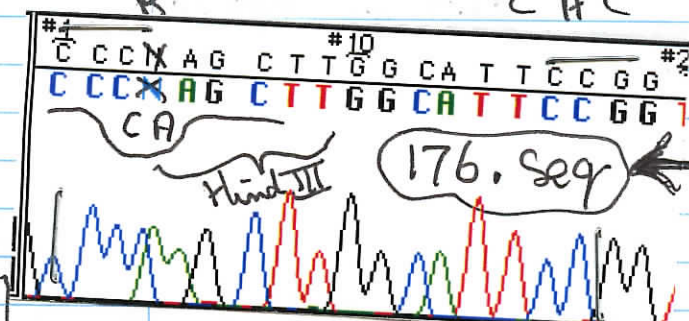


Tomoko's gel
look like self-
closure

III
↑↑↑
A B C

L79

↑ R A C A C A C Nodigestion.



Confirms that Hind III¹⁵ on 3' end
→ is okay but leaves ¹⁵ M10
or other enzyme.

176. seq
Seigs 1 June 21
Blue BMHC
with BMHC PRO
unmodified

177. seq
Seigs 2 June 21
Red BMHC

177. seq confirms that the
5' end is as expected and recorded in bMHC pro GL3. seq
KpnI PstI Bam HI → etc.

Red BMHC
L34

61 -
A
121 -
T

3421

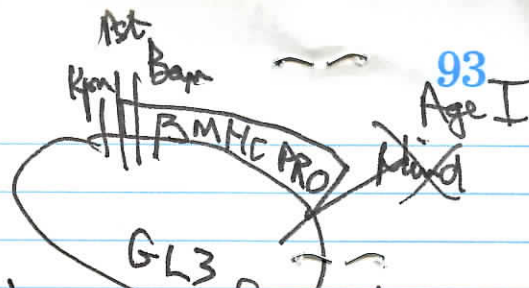
3481

3541

93
Age I

Fri June 15th

[Step (1)] BMHC PRO (cont'd.)



Tamko processed 24 from L'79 transformation. None are correct but at least 4 are not starting. Do not do more from this ligation; they are probably self-closure. ~~Reset up~~ Reset up ligation with ↑ amount (3X) of linker

Take 5 µl L'79 frag. BMHC ssH
 ✓ 12 µl L'34 Hind Age Hind oligo
 ✓ 2.1 µl 10x p95 buffer
 ✓ 2.1 µl K'sq T4 ligase

L'93
high

Cold 5 pm Fri. Transform on Sunday or Saturday.
 Fri 6 pm - 7:15 15° then back to cold
 Sat R.T. 1 pm to 2:00 pm 37° 20' to 3:05 pm → Cold
 Transformation

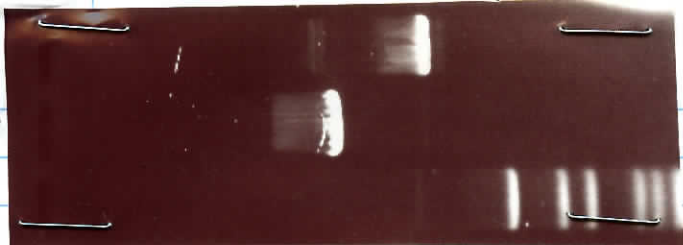
K'5.3 DH5 2 80 µl R20 5 20 5 37° 4:10 pm
 L'93 high 5 µl 16 16
 00 3:07 pm ~ 4 pm
 90 sec 42° (30) (20) (6) (0)
 L'93 minus (1) - (24) 1x Amp NZY 37° 4:40 pm - none detected
 L'93 minus (2) - (48) set up Mon. June 18th - still none!!

Tue. June 18th

Back up sequencing:

BamHI
 CTGCAGCTGGATCCCTGATGTTGGTGTCTCTGGGATAAAACCTCCCAATGCAAGTC
 GACGTCAGACCTAGGGACTACACCACAAGGAACCTATTTTGAAGGGTTACACTTCAG
 60
 BbsI
 TCTTGCGTGTGATGTGTGCTAGCCCTCACCCTGGGAATAGTCTTCATAATCCTTGGAA
 61
 AGAACGCACGACTACACAGATCCGGGAGTGGAACTTATCAGAAGTATTAGGAACCTT
 L'93 Red BMHC
 AAACCCACCTTGACAGCAAGCTGGCTCAGGGAGTTAAGTACTGAGCTAGACCACAA
 121
 TTGGGTGGAACTGTCGTTTCGACCGAGTCCCTCAATTCAGTACTCGATCTGGTGTGT
 180
 AarI BspMI
 AATCTGGCTAGGAGCTGGGTGCAGGTGGGGGATGGGGCACCCCTGCTGCCCCATATATACA
 3421
 TTAGACCGATCCCTGACCCAGCTCCACCCCTACCCGTTGGGACGACGGGGTATATATGT
 3480
 Tth111I SexAI BsmI
 L'93 Blue BMHC ✓
 GCCCCTGAGACAGGTCTGGGCTGAGCACTCTCTGCTGTTTCTTACTTGTGCCCCA
 3481
 CGGGCTCTCTCTCCAGACCGAGACTCGTAAGAGGAGACAAAGAAATGAACGACGGGGT
 3540
 AGCTT
 3541
 TCGAA
 Adding Age I here

L'93 Blue BMHC				
22	TCT	GAG	CAT	TCT
45%	GCT	GTT	T	
L'93 Red BMHC				
20	TAG	CAC	ACA	TCA
50%	CGC	AA		



L'95 Prc gel on L'90

L'95 Age II

Cut is complete
Proceed 1:20 PM

(1)

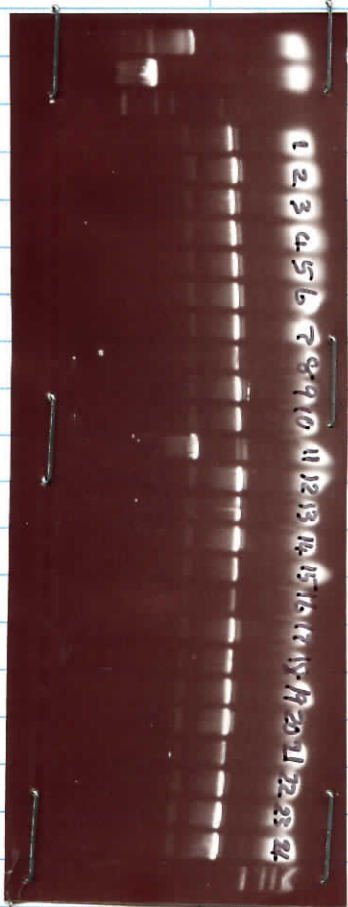
L'103

Dye
Pre
Post K
AG
(1)

L'95³ minis / Spe

(24)

PCR
Dye



} Kpm cut is okay for L'103

L'95³ #11 is okay

L'95³ (11)
YN Spe

Take $\sqrt{12}$ pl L'95³ #11 YN Spe mini

$\checkmark$ 24 pl 10X RI salts

$\checkmark$ 189 pl LHW

$\checkmark$ 2.5 pl (25 μ) Spe I Lot 28 (100 μ /pl)

$\checkmark$ 12.5 pl (25 μ) Age I Lot 33 (20 μ /pl)

37° 3:56 pm - 5:57 pm
man.

? maybe sh'd have used quickgel c.f.

K'159 with RNase!

Did bet I could with pre RNase

on the gel slice for 20 min. at 55°

prior to usual melt

Recovered into 60 μ as

Cont! on L'107

CAUTION
see L'151

L'95 YN Age
Frag^e YN Spe

(2)

Dye

W

12 slots

W

Dye

W

12 slots

W

Dye

W

12 slots

W

Dye

Sun June 24th L'95

Take $\checkmark$ 50 μ 10X RI salts

$\checkmark$ 445 μ LHW

5 μ (50 μ) Spe I Lot 28 (100 μ /pl)

20 μ aliquots + 1 μ minis

Man L'95³ (1)-(24)

Minis (1)

(24) Black

37° 6 pm

9:30 am

(1X KAN)

Sat.

June 23rd

0.5 μ RI

day

* Rel

* If un

Note: Afl II is difficult to ligate

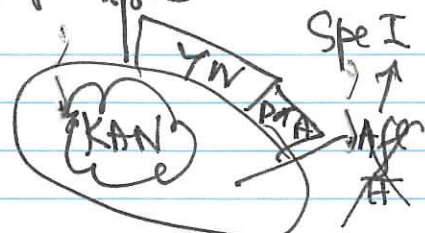
KAN

Sun. June 17th

Nancy's birthday! Age 1

95

YN per A [Seap VIII]



Take 3 μ l (1.5 μ g) pEYFP-Nuc Clontech K'119
 16 μ l L'95 PRE
 6 μ l 10x NEB 10x buffer
 1/2 μ l (5 μ l) Afl II hot 33 (10 μ l) 1997

37° 2:12 to ~3:25pm → 37° 5:10pm

Heat inactivated 10 minutes ~80°

L'95 Afl II YN

ligation:

L'95 L'95 YN

Take 3 μ l L'95 Afl II

+ 6 μ l L'88 Afl II Spe I Afl II oligo

+ 2 μ l p95 10x lig. buffer

+ 2 μ l K'59 T4 ligase

15° 10 min to 3pm RT 3pm to 3:50pm 37° 3:50pm to 4:35pm

KAN

Tanoko ran 48 - all negative. Try again with fresh ligase & NM p95 10x & 4 μ l of oligo.

L'952 Lig YN Spe

Take 10 μ l H₂O

4 μ l L'88 Afl II Spe I Afl II oligo

2 μ l p95 10x lig. buffer NM L'99

3 μ l L'95 Afl II YN

1/2 μ l L'99 T4 ligase conc

15° 4:20pm

6pm to cold

Fri 5:15pm Transformation (see L'97)

Very dry plates

6pm to 37°

(No colonies at all)

5/2/01

Transformation

YN L'953

L'97 DH5d 80 μ l

1x KAN 1x NZY

L'95 2 μ l

3 μ l R 20, 5, 2%, 5/16

Sat. 0° 10:54am to 11:20am

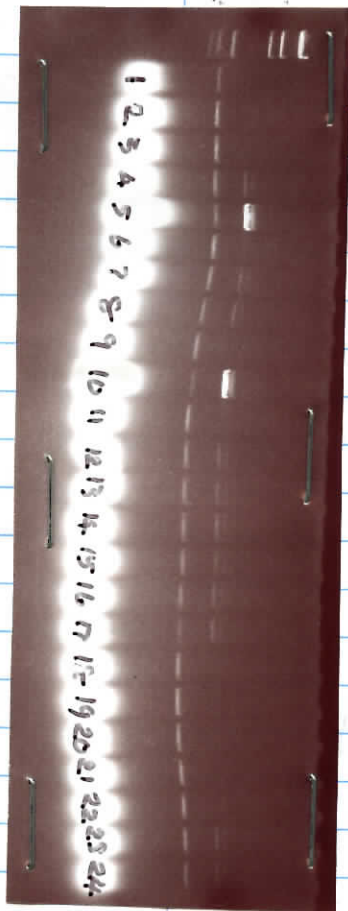
90sec, 420

37° 11:30am Sat

Sun 7:30am

many 11 2 6 10 am Sun

* Repeated - If unsuccessful - use gold cells *



T.H. gel #'s 5 & 10 are positive

1973
PUROBSP
5

1973
PUROBSP
10

3 1 $\frac{xy}{a}$

AMP

Sun June 17th

Age Puro Tap ~~Exam~~
 Amp

Repeat Step (iii)

✓ More unreacted. 20 min at $\geq 80^\circ$ for L187

negation

- Take 5 μ l (0.2 μ g) pPUR Bam L'87
- ✓ 12 μ l (3X) L'70 Bam Rsp Bam site
- ✓ 2 μ l p95 10X ligation buffer
- ✓ 2 μ l K'59 T4 ligoase

Blue  A hand-drawn diagram of a blue bag with a handle. The bag is rectangular with a handle on the left side. A label is attached to the front of the bag with the text: L'97, hīg, PURO Pst.

15th 6:12 pm to 9:05 am → ~~RT~~ RT

Tomato ran 24 - all negative -

Try again with new ligase, NM p95
 & 4 pl of oligo.

Red 7

L'972

Liq^m PUROBsp

✓ 11 µl ddw
Take 3 µl (NO. 1 µg) pPUR Bam L' 87
✓ 4 µl L' 70 Bam Bsp Bam ligase
✓ 2 µl p95 10x lig. buffer NM L' 99
✓ ~ 1/2 µl L' 99 T4 ligase NEB 4000 µl

15^o 4:20^{Thurs} pm

6 pm to call

(L'97)

$$L'_{95} + L'_{101}$$

old way
I want/pol

→ Transfompha (also $L_{95} + L_{101}$)

L'97	DH52	80	pe	1xNZY	1xAMP
------	------	----	----	-------	-------

L'972 h₅ⁿ

$R_{20,5,29/16,5/16}$

Fr. ∞ 5.15

90 sec 4.2°

37° 6:05pm

~ 4/11 ~ All were 3 pl

Colony numbers were okay

Tomoko picked 24 to 37° at 4 pm Saturday

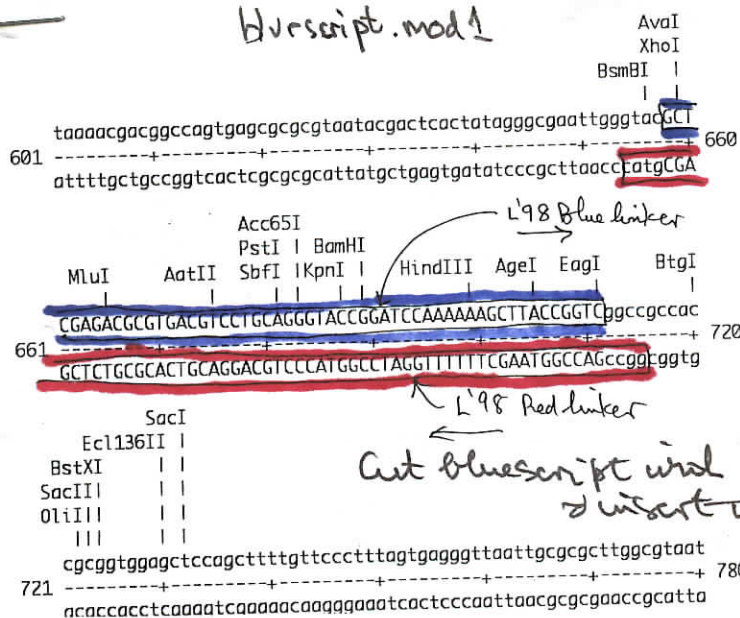
Not so well ~~at 20~~ ~~as before~~ aerated

L'973 Minis (1) - 24

PURC 6/23 TH (TH. well test)

Red

bluescript.mod1



Enzymes that do cut and were not excluded:

AatII	Acc65I	AgeI	AhdI	AloI	AlwNI	AvaI	BamHI
BcgI	BsaI	BsaAI	BsmBI	BstXI	BtgI	DraIII	EagI
Ecl136II	HindIII	KpnI	MluI	NaeI	NgoMIV	NspI	OliI
PciI	PsiI	PstI	SacI	SacII	SapI	SbfI	ScaI
TatI	XhoI	XmnI					

Enzymes that do not cut:

AarI	AccI	AfeI	AflII	ApaI	AscI	AvrII	BaeI
BbeI	BbsI	BbvCI	BclI	BglII	BlpI	BplI	Bpu10I
BsaBI	BseRI	BsgI	BsiWI	BsmI	BspEI	BspMI	BsrGI
BstAPI	BstBI	BstEII	BstZ17I	Bsu36I	BtrI	ClaI	EcoNI
Eco0109I	EcoRI	EcoRV	FseI	HincII	HpaI	KasI	MfeI
MscI	NarI	NcoI	NdeI	NheI	NqtI	NruI	NsiI
PacI	PflMI	PmeI	PmlI	Ppu10I	PpuMI	PshAI	PspOMI
RsrII	Sall	SanDI	SexAI	SfiI	SfoI	SgfI	SgrAI
SmaI	SnaBI	SpeI	SphI	SrfI	StuI	StyI	SwaI
Tth111I	XbaI	XcmI	XmaI				

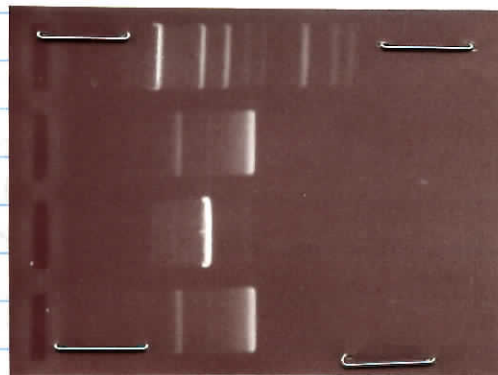
① clearly, there is no Mlu site
 ~ confirmed by L'92 sequence

Oligo Name: L'98 Blue linker

48	GCT	CGA	GAC	GCG	TGA
58%	CGT	CC-T	GCA	GGG	TAC
	CAA	AAA	AGC	TTA	CCG
	GGT				

Oligo Name: L'98 Red linker

56	GGC	CGA	CCG	GTA	AGC
61%	TTT	TTT	GGT	ACC	CTG
	CAG	GAC	GTG	ACG	CIGT
	CTC	GAG	CGT	AC	



by

BMC PRO

99

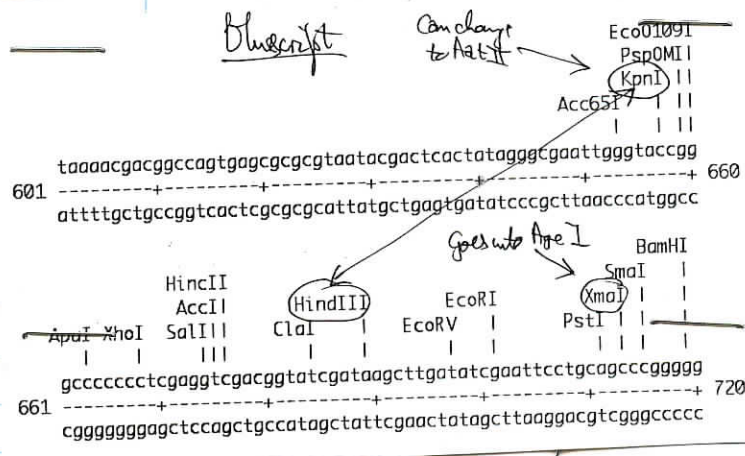
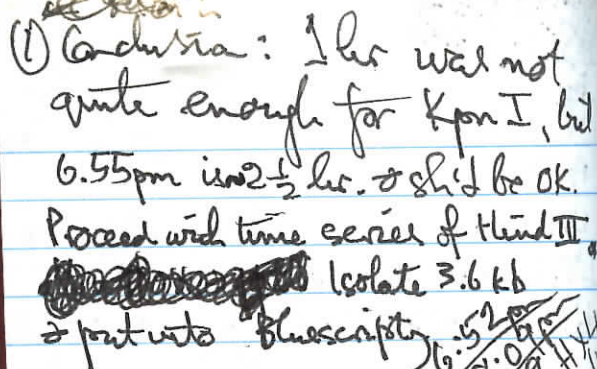
L'996 BMHCPROMIV Can expect to excise BMHCPRO w/ ~~the~~ Kpn or Bam or Pst from single cut then lig and preparative gel followed by cloning into modified Bluescript that will allow excision of (ii) & (vi) directly. But the cut is still very difficult. An alternative may be KpnI and NotI → Age I

11114 ngase

4.5 M HCL 3
AN 1304
gel
10:25 am to 10:36 (10) 37°
Heat unact. at 78° 10

L'99 T4 tugas 5 Bilahs 4000 / pe
lot 71 7

5 Bidsaks 4000 / pe
het 7!



② Conclusion:
clearly the
luggage is the
likely problem.

Several candidates:-

8, 12, 13 & 16. Test with Age I
alone & sequence with L'93 Blue primer

13 & 16 (T.H. 6.25.01) & 15 out with Age I

3.5 kb expected
& ~5 kb

Agent 3550

$\swarrow$ $\downarrow$ $\nwarrow$ can see on my gel also
 $\swarrow$ ~ 350 bp $\nwarrow$ Age at 350
 $\nwarrow$ ~ 8 kb

12
13
15
to sequence with
L'93 Blue

See L111
Case

Both
w/ Hind
3 Hind → Hind

#16
with L'93
Blue

Hind

179.8g

#13

with 13

Blue

1:15 pm
 1:25 pm
 0.8% H₂O
 Max
 Dye
 W
 L'101 Pre

Fri. June 22nd

Age I
 101
 H₂O

Backup for Stepli again

(AMP)
 (3MHC PRO)

H₂O
 3% RWA
 MAX

L'101 Kpn Trying to fix ligase etc.

✓ 11 pl L₂W
 Take 3 pl L'79 Frag⁺ BMHC ss H
 ✓ 4 pl L'34 H₂O & Age Hind oligo
 ✓ 2 pl p95 10x lig⁺ buffer L'99 NM
 ✓ 1/2 pl L'99 TA ligase conc

L'101
 L'10 W
 MHC Age

Blue 15° 9:45 am

L'101 minis (1) - (24) (20, 22, 23, 24) low air-dried

1.40 pm DT - 3:10 37° 3:10 to 3:55 pm

Fr. 5:15 pm Transformata (see L'97)
 2 auto Amp. 37° at ~6 pm Fri. Colony numbers
 were okay. Tanks picked colonies. Tol 37° at 4 pm Saturday. Let 20 tubes air-dried

Also take 10 pl (? 2 pl) { BMHC L'101 }
 + 15 pl 10x RI salts
 + 125 pl L₂W
 + 1 pl (200) lot 50 (200/pl) Kpn I
 37° 4:30 to 5:37 pm → 37° 6:55 pm Frizer

Hold pending minis

Cancel

Take whole of L'101 BMHC Kpn, diluted with ~~water~~ 110 redddy 12 pl 10x RI salts
 & warm to 37°
 Add 0.5 pl (10 u) Hind III lot 52 (200/pl)
 Aliquots of 450 pl to 80-85° for ≥ 20 min
 "0", 1, 2, 4, 8

Take ✓ 50 pl 10x RI salts
 ✓ 422.5 pl L₂W
 ✓ 25 pl Age I (500) lot 33 (200/pl)
 ✓ 2.5 pl Bam HI (500) lot 82 (200/pl)

** ✓ 1 pl L'101 minis (1) - (24) (Blue) no #21.
 Sam 37° 5:16 pm to 6:35 pm

↑
Nat↑
Aat + Bsp(L'91
gel)

Kpn + Hind III

Recovered as

L'103 w/ NctA Aat/Bsp Fragt

① The GL3 Kpn didn't work. Repeat in a better manner w/ StOH ppt. after pot. Cut for Aat II / Bsp I fragt. is good. The two Aat/Bsp smaller fragments are correct.

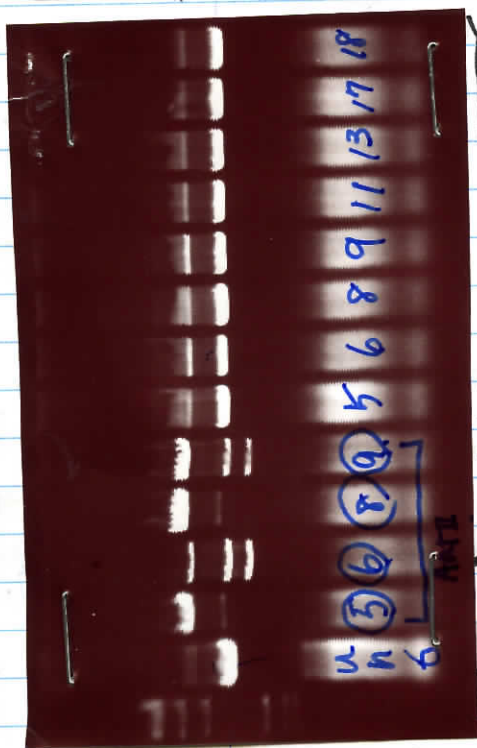
~~Yes Aat~~
BMHCP



Gel on L'101.

Pre (iv)

Backbone



T.H. gel with

Aat III

← ⑨ } Aat II
← ⑥ } + Age I

No enzyme

15° 12:

didn't
2
wed
pOH
spEI
he
R⁺ 37 kb

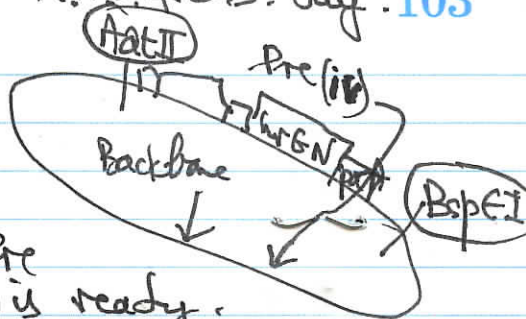
BMHC PRO

Sat - June 23rd.

Pre
Step (iv) repeat (L'91 cont!)

Recovered into 75 μ l 0.001M-EDTA. Pre
Product is fine & good recovery. (iv) is ready.

RWS & O.S. day 76th 103



Fragt

Mon. June 25th

Step (i) BMHC PRO
Kpn $\rightarrow$ Aat

Take 70 μ l L'101 Mini (16), # (i) candidate

✓ 128 μ l ddw

✓ 15 μ l 10 $\times$ RI salts

✓ 1 μ l (20v) Kpn I hot 50 (20v/ μ l)

37 $^{\circ}$ 9:25^{am} to 11:55^{am}

L'103
PRE

L'103
Kpn BMHC
CIAA

16

✓ cut is good. Gel on L'94.

ϕ OH/HOQ 2 $\times$ CIAA

ligation

L'103
POST Kpn

Take 1 μ l ddw

blue

L'103
BMHC Aat
Lig

✓ 3 μ l L'103 Kpn BMHC CIAA

✓ 4 μ l L'58 Kpn Aat Kpn oligo

✓ 2 μ l p95 10 $\times$ lig. buffer (O.S.)

✓ 1 μ l L'99 T4 ligase conc.

15⁰ 12:30^{pm} to 3:15^{pm} PRT to 4:30^{pm}

5:10^{pm} Transformation $\rightarrow$ Gel

1 $\times$ AMP NZY

L'97 3/13 DH5 α ✓ 80 μ l

L'103 lig. ✓ 3 μ l

0 $^{\circ}$ 5:15 to 5:45^{pm}

42 $^{\circ}$ 90 sec

R, 20, 5, 2d/16, 5/16 37 $^{\circ}$ 6^{pm}

Adequate numbers Tues.

To T.H. for making minis

June 25th
Monday

~~Need to repeat since this was in error~~

L'103 T.H. 4 page 7 6/27 #6 and #9 are clearly modified.
Continue with L'103 6 MHC Aat. Photo on L'102.

104



15

L'94

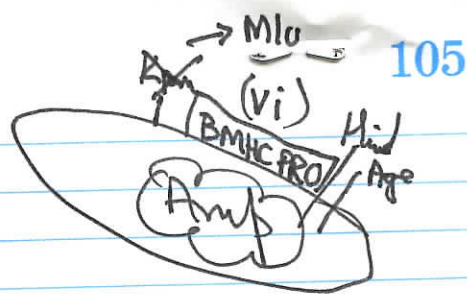
(100)

3

xy
all

Mon - June 25th

Step (vi) BMHC PRO
Kpn → Mlu



105

Ligation Take 10 μ l ddw

- ✓ 3 μ l L'103 Kpn BMHC CIAA
- ✓ 4 μ l L'70 Kpn Mlu Kpn oligo
- ✓ 2 μ l p95 10 $\times$ lig⁺ buffer
- ✓ 1 μ l L'99 T4 ligase conc.

Red
L'105
BMHC Mlu
lig⁺

Mon.
15⁰⁰ h:15 pm

~~overnight.~~

Transformation

L'97 3/12 DH5 α

L'105 lig⁺

00 1:45 pm to

90 sec 42°

1X AMP NZY

80 μ l R 20, 5, 2, 5/
3 μ l many (16) (16) (20)

Tue.

3⁰⁰ 3 pm

To T.H. for making minis.

~~Need to check if ligase is correct~~

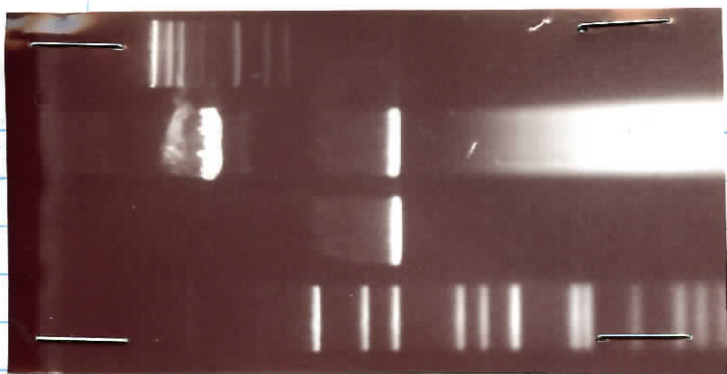
L'105 Pooled

Bulk prep⁺ made from (15)

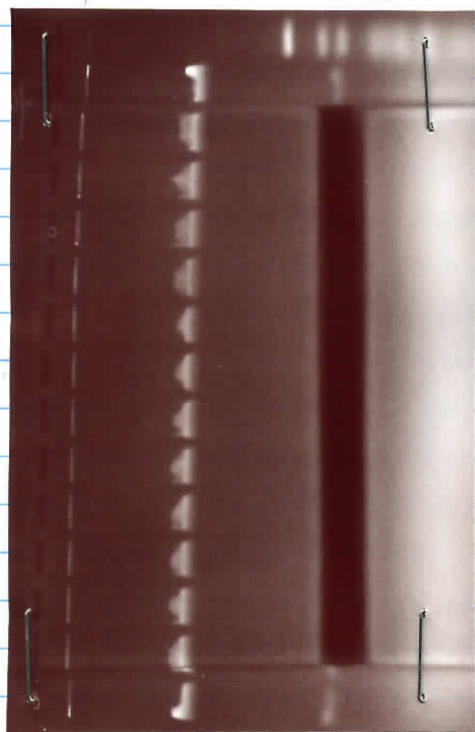
of Mlu/Age

& kept L'105 (15) unpooled.

106



(viii)
L94
① The fragment (viii) is
in excellent yield &
high quality. Size
predicted is 1070 + linker's
size observed is ~1070bp.



② good cut.

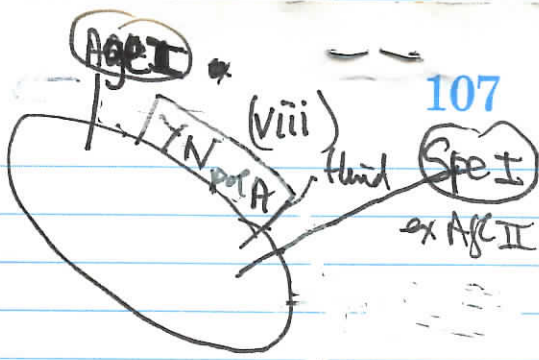


③ (iii) is good!

145
170
0.80
Hx4

194 Hx2
160 Hx2
1232pm NAK
(8 V initially)
Sealer down to
Tues. June 26th

(Viii) Age I YN pol A Spe I (Contd.) L'94-95

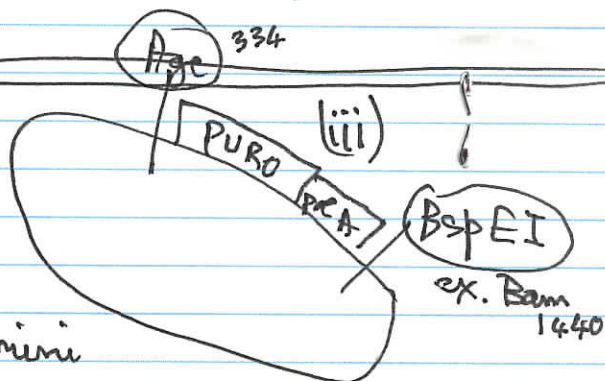


Back up cancelled!

Ag
W
Ag
L'94 YN Spe Age
L'95 Frag^t YN Age Spe
ACR
W
Dye

5:20 PM
10:11:18 AM
RNAse
3µl/10µl/cm
1/2 V
Tues. June 26th

(2) ACR (ii) Age/Bsp Puro pol A (L'97)



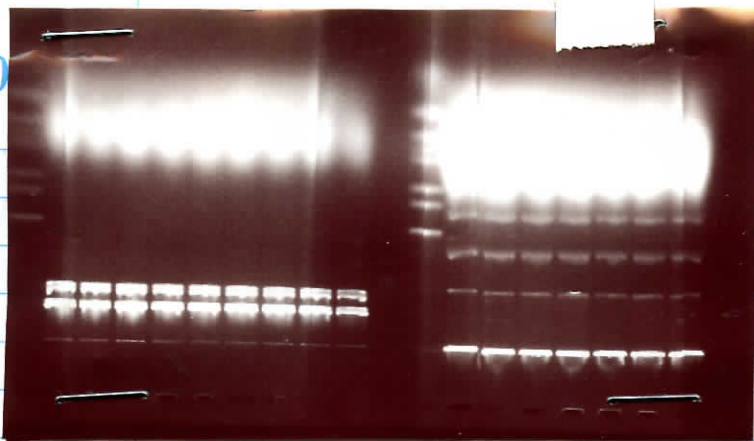
Seab
0.8% Hx4
Take 12µl L'97³ (10) Puro Bsp mini
✓ 24µl 10x R1 salts
✓ 189 µl L'107
✓ 12.5µl (250) Age I Lot 33 (20/µl)
✓ 2.5µl (250) Bsp EI Lot 3 (100/µl)
37° 2:38pm to 4:55pm

L'107
Puro
Age Bsp
ON 4:12pm
5pm 2xH
1.6% 1/10cm

Back up into 50µl at L'107 Puro pol A Age/Bsp
Frag^t (iii)

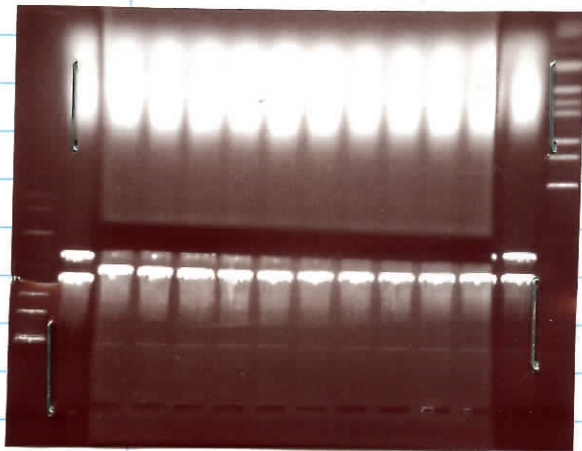
(3)
Bsp
W
ACR
L'107 Puro Age Bsp
L'107 Frag^t
W
Dye

10



(1)

(viii)



(2) Good cut for the 3.5 kb fragt
 L'103) AAT BM HC PRO Age
 L'109 Fragt (ii)
 Back into bone

on 5

0.8

RE

Wed. June 27th

109

Fragment (X). hrGN pol A

Take 12 pe L'109 hrGN CTA AAT II

✓ 24 pe 10xR1 cells

✓ 185 pe Ldw

✓ 12.5 pe (250) Age I Lot 33 (20/pe)

✓ 6.3 pe (250) Age II Lot 29 (40/pe)

hrGN Aat
L'109
Age/Aat

37° 2:58 pm - 5:52 pm to freezer

Thurs. June 28th

0.8% Hx4 dye water

PCR MM

0.75 V/cm

~10 slots L'103 BMHC (6) Aat/ Age

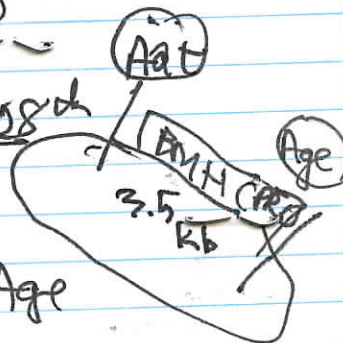
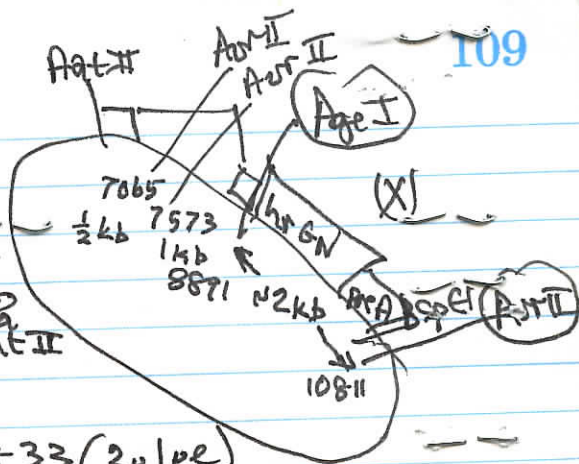
dye

ACR MM

~6 slots L'109 hrGN Aat/ Age/ (Aat)

Slots were rather big - gel had partly dried.
Do best I can.

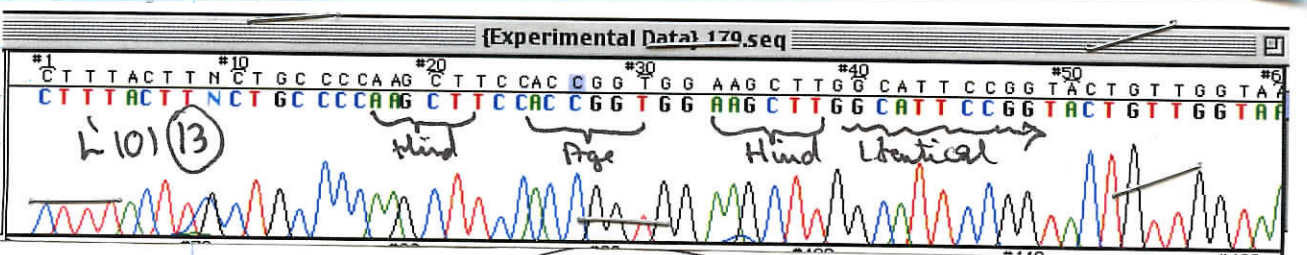
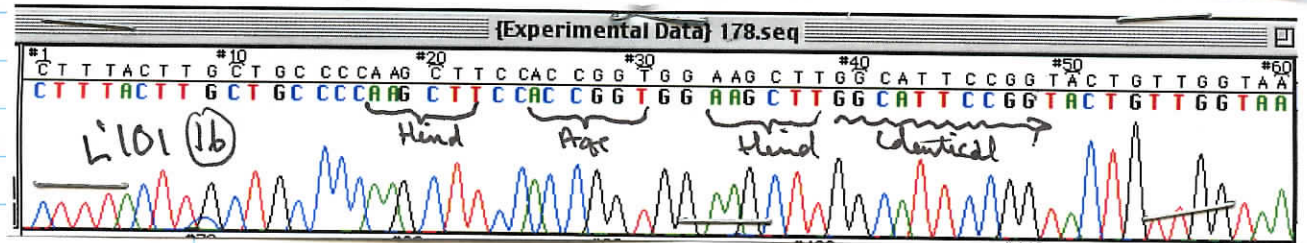
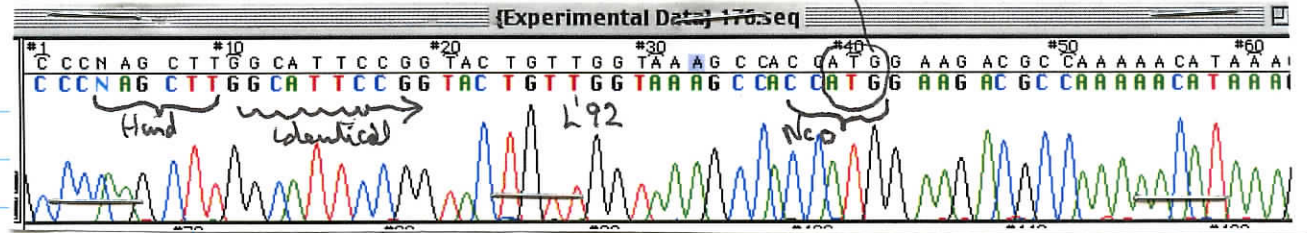
- ① After staining, I misinterpreted the BMHC result - it was ^{really} okay - and discarded the gel. The hrGN pol A digest was not complete - T.H. will repeat. Meanwhile repeated L'103 BMHC (6) Aat/ Age with 12 slots AG ~~XXXXXXXXXX~~ AC at 3 V/cm. On 1:20 pm
- ② off 4:25 pm Yellow ~ 8/10



(vii)
over

(viii)

Danger Met



L'101 data L'99

Cancelled

Wed. June 27th

Back to GO etc.

Step (i) again

Transformation

L' 97 DH5 α 80 μ l

L' 101 lig. 3 μ l

0° 6:20 pm to 7:20 pm

42° 90 sec

Plated 1X Amp NZY

Many (20) (5) (0)

R, 20, 5, 50, 5/16, 5/16 37° 7:30 pm

L' 111
MHC Age

9:30 am

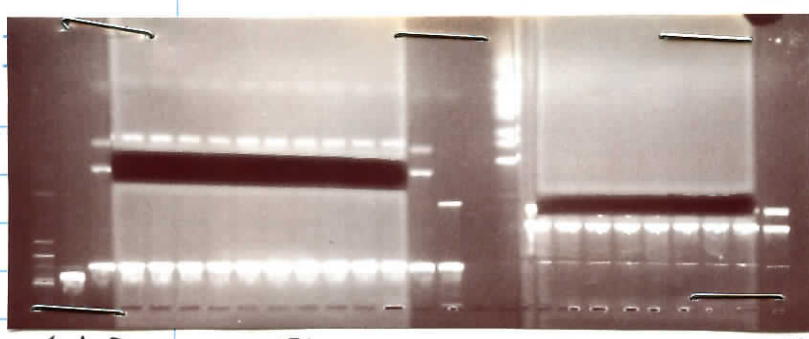
Cancelled
because of

Interpretive error due to using a June 21 (incorrect) version of BMHC pro GL3 seq. instead of later correct version June 27. There was a ~50bp Hind III fragment that appeared to have been lost from the June 21 version. Netblast identified it as a portion of GL3 incorrectly placed during assembly. The correct version BMHC pro GL3, 176 seq. (unmodified plasmid) → 178 / 179 sequences (post modification) are in agreement — Age I had been correctly inserted into the deleted 3' Hind III site of BMHC promoter prior to the NCO (ATG).

(vii)
over

(viii)

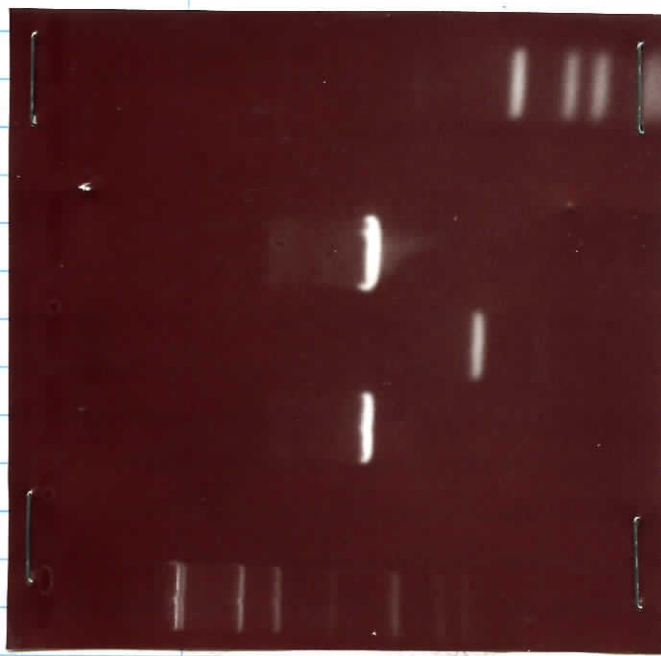
508
1318
1920



1 Age Supplant OK
Not II Three cuts OK
Age + Age Four cuts OK
→ ~~Band~~ Band (slow) at
~2 kb isolated (1920/6)
Mlu/Age BMHC PRO
- the 3.5 kb band isolated

(X) Back into 50 µl
as L'113 hr GNpoeA
Frag² Age/Age (X)

(vi) Back into 50 µl
as L'113 BMHC PRO
Frag⁵ Mlu Age (vi)



2 Looks good!

← ^(Age) BMHC PRO ^(Age) (i) L'109
← ^(Age) hr GNpoeA ^(Age) (X) L'113
← ^(Mlu) BMHC PRO ^(Age) (vi) L'113

Hx4
0.8%
Ag
+ RNAse
(X)

(vi)

OM
(2)

Undil

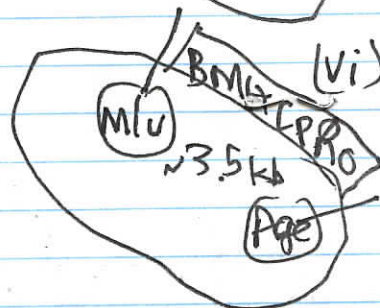
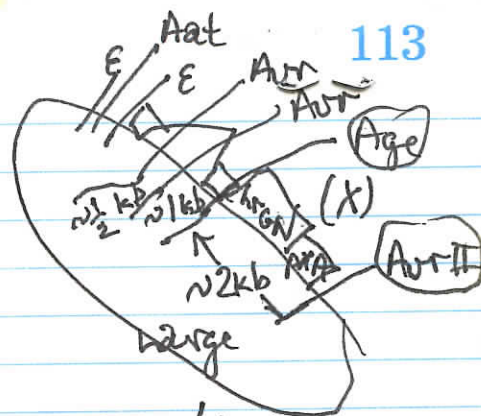
Friday June 29th

(X) Age hr GN pre A (Aut)

T. H. Set up Age, Age+Aut, Aut with hr GN CTA. Aat

(1) and M/U + Age for M/U BMHC Age (vi)

Hx4 0.8% Age A/GMM 0.8% Hx4 Scap + RNase
 Age L'113 - - -
 hr GN Age+Aut
 L'73 - - -
 Aut
 Dye
 ACR
 (vi) L'125 M/U BMHC Age ← Red
 PRC
 Dye
 0.5 V/cm to 10:15 am



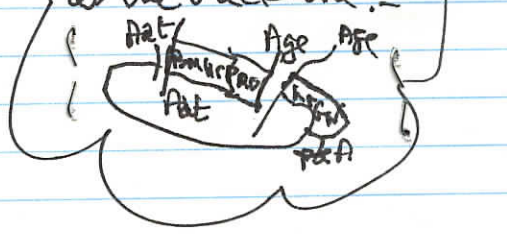
on 3:06 pm 0.8% max 5V/cm Fragment text Sat. June 30th

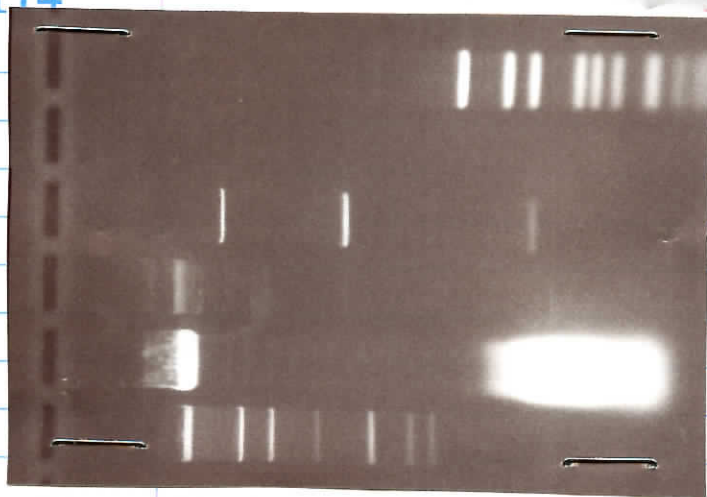
(2) Dye W ACR W (ii) L'109 Frag^c BMHC PRC Aat Age (X) L'113 hr GN pre D Frag^c Age Aut (vi) L'113 BMHC PRC Frag^c M/U Age W AG W Dye

? Could have made Pre(iv) at same time!

Comment

(X) was not the best for this, since it had extra sequences (human HPRT) & could have used Aat anyway as the backbone:-





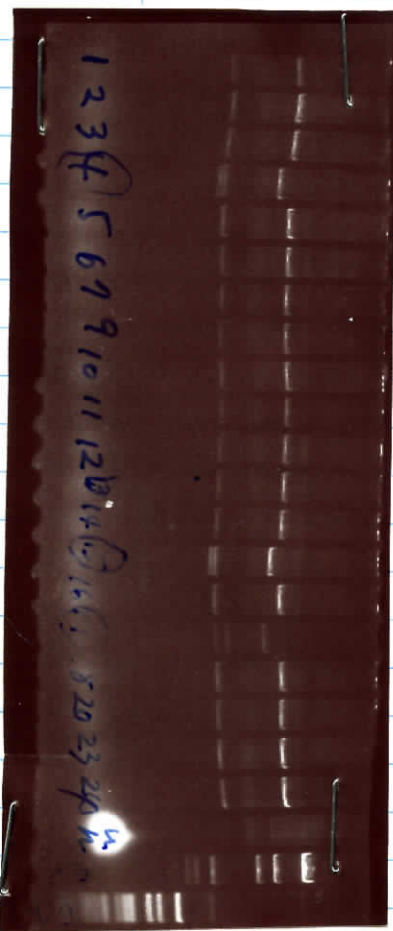
① Fine prepod trans. good
ligase! L'117 Aat cut is complete

See M

Most likely conclusion is that
Aat II did not cut. T.H. does not
work minus with 0.25M Tris & the
enzyme is v. pH dependent.
Re-cut #15 & 16 with EcoRI & BspEI

②

3 Bam sites



Revised conclusion:

(4) (15) & (17)

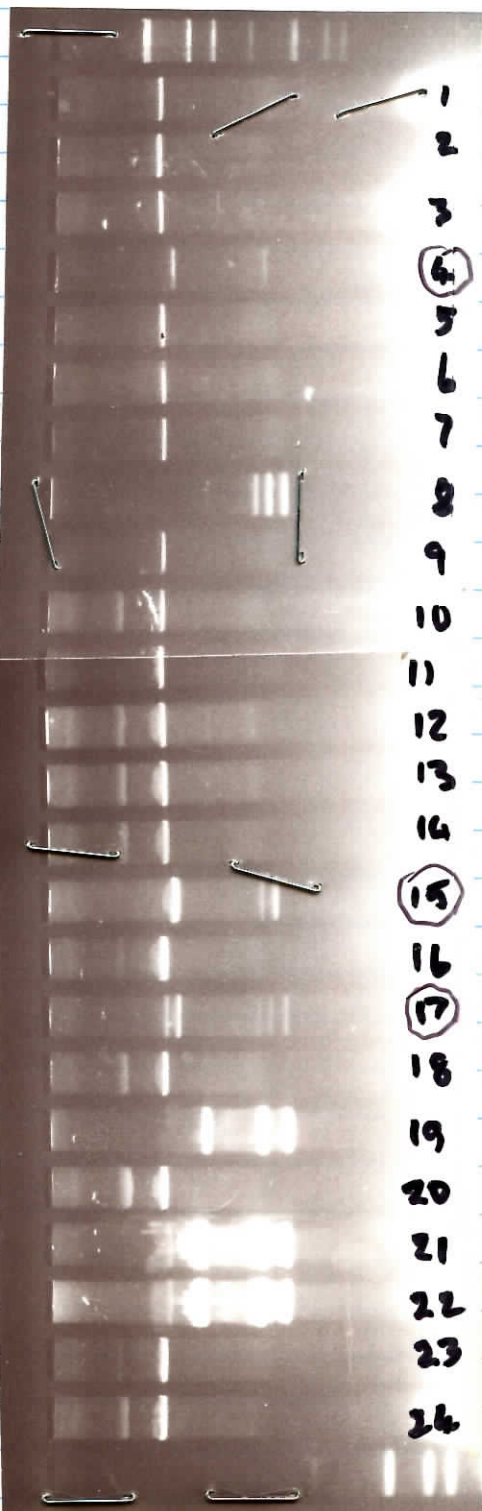
may have Aat II
sites and
3 Bam sites.
So Aat
boundary
may be
partially
correct.

Tonako Bam + II digests.

(15) (17)
is
most
likely,
just
from
Bam

3 Bam sites

Continue with
L'115 Red (17)



L'115
XAMP

No

See M
for page

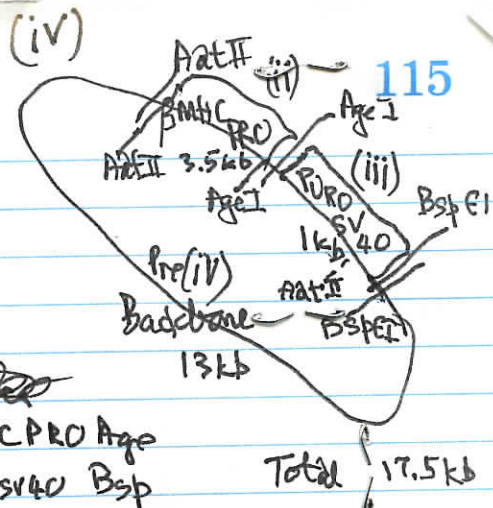
complete

On 12:40pm
On 2:00pm

Now called **L'115 BMHC . PURO**
(was Red 17) Sun. July 1st

First assembly # (iv)

BMHC PRO . PURO SV40



L'115 AatII high
L'115 Post high
L'117 Post AatII

L'115
W

Take ✓ 10 µl L'109(ii) Aat BMHC PRO Age
✓ 20 µl L'107 (iii) Age PURO SV40 Bsp
✓ 10 µl L'105 Pst(iv) Bsp

✓ + 4 µl 8M NH₄Ac
✓ + 110 µl EtOH

On 5:15pm
0.81 g x 4

Back up into 20 µl 1x p95, then 5 µl to 1 µl
✓ Rere + 1.5 µl L'99 T4 ligase conc

PRE
L'115

Age
W

15° 21' to 5:00pm RT 3:10 to 6:26pm 32° 6:26pm to 7:03pm → Cold

①
②
ACR
W
Dye

L'115 minus
AatII + BspEI Transformation

L'97 DHS α
L'115 high

80 µl
3 µl

1x AMP NZY

R, 20, 5, 20, 5, 16, 16 37° 7:35pm
many (720) (8) (0) Sun 2pm Mon

L'115 Minis ① - ②4 BMHC PURO

AMP NZY 37° 3:40pm Mon.

T.H. prepared → ligated
- ending at 4:50pm

Note AatII cuts (PURO) at 610 bp 3' to AgeI. Eco RI & Bsp EI do not cut. None of these cut (BMHC).

Re-test with Eco RI and BspEI in RI salt.

None cut, but Bam digest suggests that (17) is correct, despite the Eco RI problem - see L'123 gel

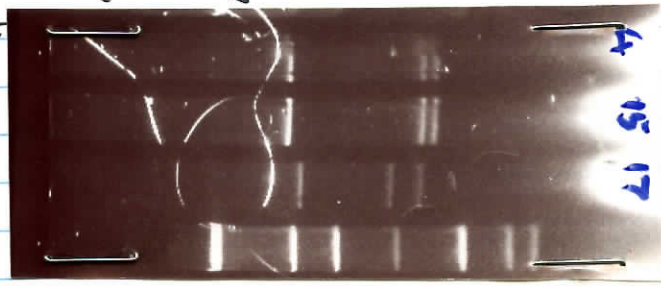
→ Bam vector backbone is correct

Predicted Bam for BMHC . PURO:

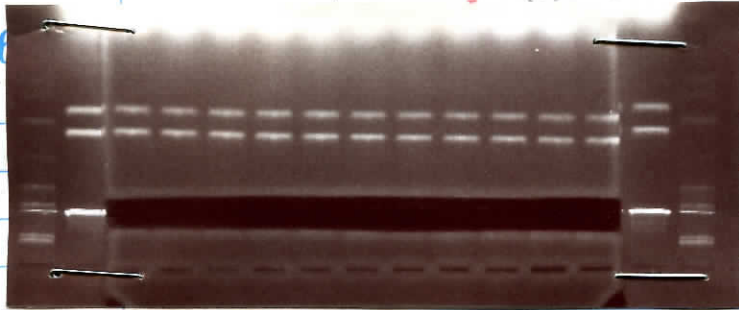
4.6 kb 3.9 kb 8 kb

See M16 for positive results

L'115 Red 17
BMHC PURO



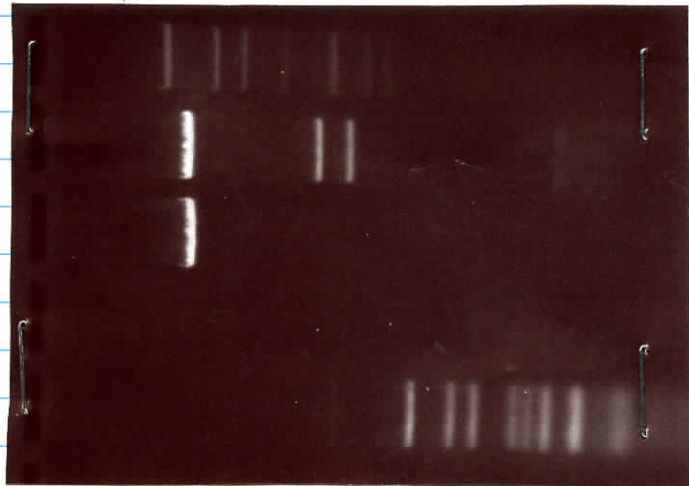
116



① Nice separation - but
on the gel-break line.
Had to use hot ($>90^{\circ}\text{C}$)
phenol to dissolve.
Into 65 μl

as

L117	AX
Fract	Fract



② Good recovery. The bot
phenol is safe.

①
2:1
on
A
1386
②



Monday July 2nd

Pre xii Art II - Art II

backbone

L109 Pore TH 3-139

Take 12 pc ¹/₇₃ hr 6NCTA mini
24 pc NEB buffer 4 10x
203 ~~ml~~ 22w

1.20 pl (24v) Aat II Lot 48 200/yr (2001)

37° 9:40^{am} to 12:20

✓ 12 ~~μl~~ ^{μl} IMNACE (10X)
✓ + 6.3 ~~μl~~ (25_u) APT II hot 29 (40 ^{μl})

27th 12:30 pm to 2:30 pm - to freezer

0.8% Hx4 Ag./Scap. 0.5V/cm Yellow 75%

(2) ~~On 12.05.2021 2:45 PM~~
~~0.89 Hx4~~
~~max 3/10 RNAcc~~

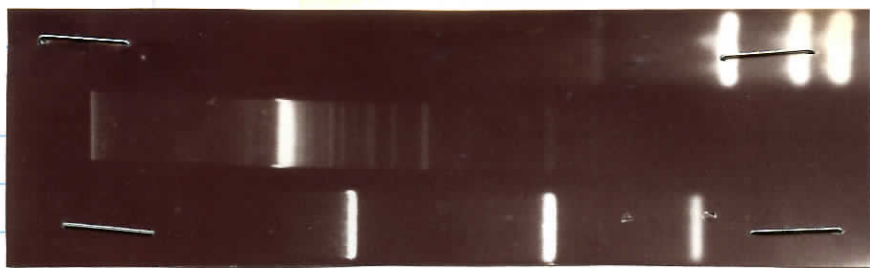
Dye
 W
 Ag
 L'117 Aat Aut load
 L'117 Aat Aut Trag I
 W
 ACR
 W
 Dye

Handwritten diagram illustrating a DNA replication bubble. The bubble is represented by two converging lines. At the top vertex is a circle labeled "Aat II". To the right of the top vertex is the number "117". Below the top vertex is "~ 4500". The left arm of the bubble is labeled "Pro xii Backbone". The right arm contains a box labeled "2.5 kb" and another box labeled "500 bp ~ 7070". Below these is a box labeled "3 kb ~ 7570" and another box labeled "~ 10810". At the bottom vertex is a circle labeled "Aat II". To the left of the bottom vertex is a box labeled "~ 13.5 kb". Arrows at the ends of the lines indicate the direction of replication. There are also some faint markings like "9", "ni", and "10X" on the left side.

O.K.
See L 115
and

L117
Parr Act

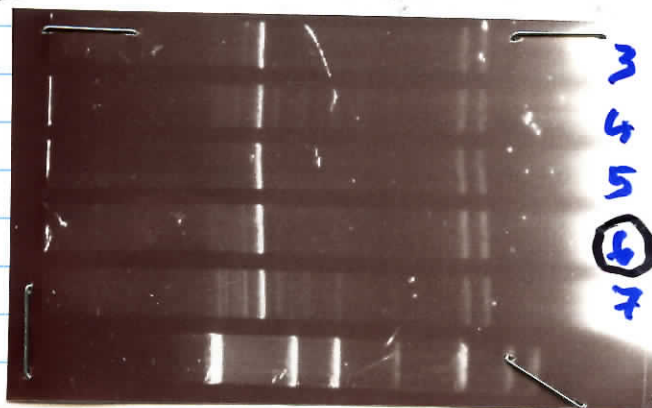
L119
Post
Pre
lig.



(Gel on L121)

Good ligation. Washed
gel shows polymers well

Conclusion: Many positives (3)(4)(5)(6)(7) Cook v. clear.
Aot II + Aot II digests. T.H.



3
4
5
6
7

L123
Re-run
of Aot/Aot
digests
of L119
minus

Continue with L119 Blue (6)

Plat
R. 20
man

T.H.
with 25%
0.25
TV

Darked
ers well.

(Xii)

Tues. July 3rd

Assembly (Xii) BMHC PRO hr GN poeA

Take 10 μ l L'109 (ii) Aat BMHC PRO Age
✓ 20 μ l L'113 Age hr GN poeA A or (X)
✓ 10 μ l L'117 (Pre Xii) Aor Backbone Aat
✓ + 4 μ l 8M NH_4A
Red + 110 μ l EtOH

L'119
WG^N

Back into 20 μ l ✓ 1x p95 buffer, then 5 μ l + 11 μ l

Rest + 1.5 μ l L'99 T4 ligase conc.

15° 2:05 PM to 4:15 PM, RT 4:15 PM to 5:35 PM 37° 5:35 PM to 6:05 PM

5+11

PRE
L'119

Transformation

Diff 5 & 80 μ l ✓
L'119 WG^N 5 μ l ✓
06:05 PM to 6:25 PM (intended 3 μ l)

5+11

POST
L'119

V. wet plates.
Plated 1xAMP NZY

20 5 20 5
many 220 16 5 16
37° 6:35 PM

T.H. made minis & washed them 4.2° 90 sec
with 25% EtOH digested with Aat II & Aor II
0.25M Tris pH 8

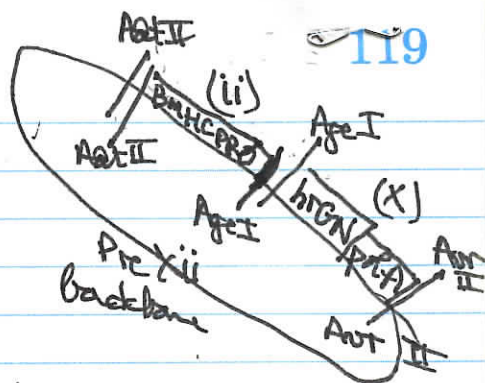
Expected sizes are :- (2.6 kb), (2.9 kb) & (13 kb).
Confirmed by gel on L'123 & photo on L'118

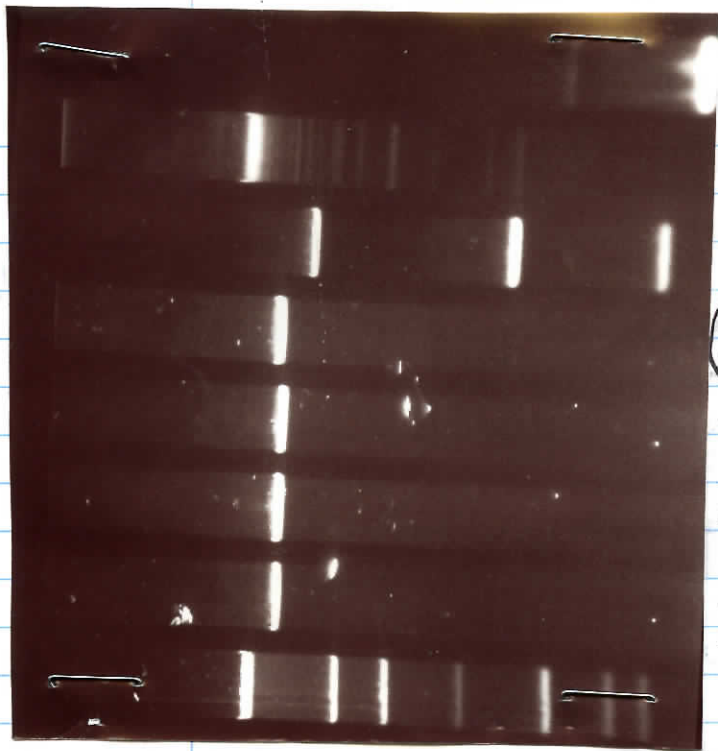
This plasmid was called **Blue 6**

and is now

L'119 BMHC ~~PRO~~ hr GN poeA

See M'17 for positive results
with it.





- ① ligation for L'119 is fine.
 No correct candidates by Eco
 in these 4 for (iv). Check
 all sites again with
 EcoRI & BspEI. If none, repeat
 ligation with more backbone
 unless sequence data is contradictory

⑤

10
23
24

①
 P-
 P-
 +

(iv)

Wed. July 4th (1)

Re-tests of BMHC PRO. PUROSV40 (iv) L'115

11:52 am
12:52 pm
0.8% HXK
max

- ① Dye
- W
- ACR
- 119 L'119 high
- 119 L'119 high
- 15 } L'115
- 16 } minis
- 23 } EcoRI/BspEI
- 24 }
- Ag
- W
- Dye

Take 10 μ l 10x EcoRI salts
 88.5 μ l ddw
 1/2 μ l (10 u) EcoRI lot 29 (20 u/ μ l)
 1 μ l (10 u) BspEI lot 3 (10 u/ μ l)

Note there are T.H. unwashed minis.

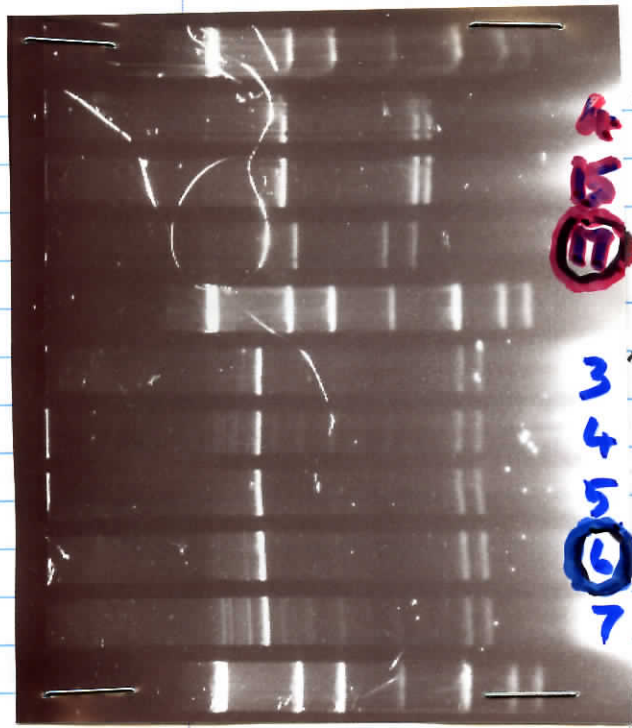
20 μ l + 2 μ l L'115 minis 15, 16, 23, 24 37° 9:22 am

Correct sh'd cut out a 4.5 kb band + leave 13 kb backbone.

To confirm sequence with K'17 Blue MLV make a new red primer L'121 Red AVR to use with either BspEI or AvrII backbone.

		L'121 Red AVR			
56	CGC	CTA	CGC	CGG	AAC
18	CTA				
67					

This primer is from 3' homology ending in AVR II sequence.



4
5
17 } L'115 BMHC.PURO
Bam ← Good

Red 17

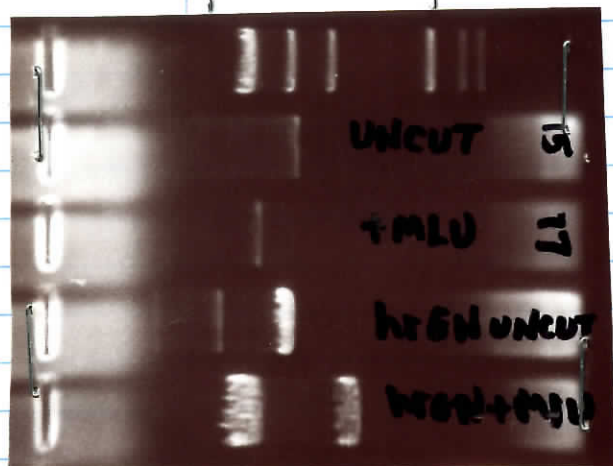
3
4
5
6
7

L'119
Not
Avr

All good

Blue 6
BMHC.hrGNLong

Blue 6



T.H. gel

UNCUT 5
+MLU 17
hrGN UNCUT
hrGN +MLU

L'115 BMHC.PURO
← (17) has an M15 site.

W 10:1
H
Dye
W
AG
4
15
17
3
2
1
6
5
4
3
2
1
V
U
H
7

Fri. July 6th

Same repeats

Take ✓ 8 pl 10x RI cell

✓ 72 pl ddw

✓ 1 ~~pl~~ (20 v) Bam HI Lot 82 (20 v/pl)

✓ 20 pl + 2 pl L¹¹⁵ mins (4) (15) & (17)
37°C 90 min to —

0.8% H₂O Max

(Poor gel)

Dye
W

AG

(4)

(15)

(17)

L¹¹⁵ mins
Bam HI digest

AG

(3)

(4)

(5)

(6)

(7)

L¹¹⁹ mins
Aat II / Aor II
digest

~~(17)~~

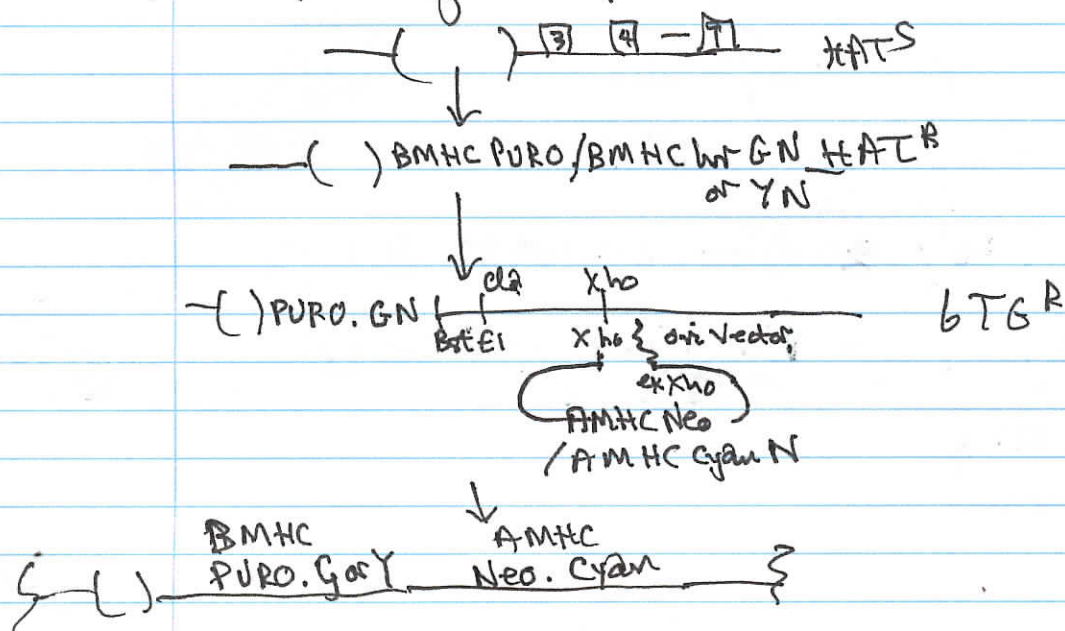
~~(17)~~

AG

W

Dye

5) A good long-term plan will be: -



3
125

Fri. July 6th

Redesign

continued by 2' 122 gal

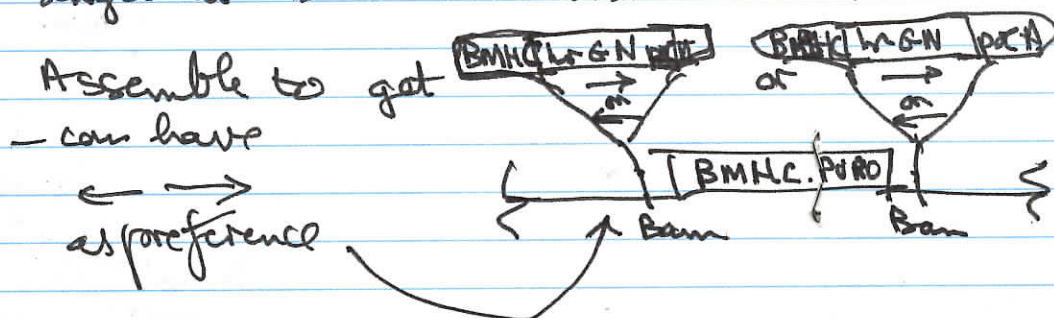
The maps of BMHC, PURO and BMHC hr GN long show that BMHC, PURO has an Mlu site (forgotten), but both show simpler routes to (iv), and

eventually to (ix). Both (iv) and (xii) short can be excised with Bam HI. Single site Bam HI on either will, at 2 out of 3 sites, ~~work~~ after CIAP treatment, accept the Bam frag^t and give (ix). Best is:—

a) From BMHC hr GN long excise the short
(Bam) BMHC hr GN (Bam)

b) Single cut BMHC, PURO and CIAP treat.

c) Assemble to get
— can have



d) Note that NG, PURO can be excised cleanly with Eco RI and Bsp FI, or with ~~site~~ Mlu & Bsp FI.

e) Note also that $\Delta HN \phi 101$ can accept Eco RI — Bsp FI into Hind 3 ~~HPRT~~ $\rightarrow$ give smaller construct. But need to find linearization site, or pre-convert one of Hind 3 sites. This shd remove unwanted sequences.

Fri. July 6th

Bam Fragment hr GN per A short

- Take 12 pe L'119 BMHC PRO. hr GN long mini (6)
- 12 pe 10x RI salts
- 95 pe ddw
- 1 pe (20u) Bam HI hot 82 (20u/pe)

L'127
(6) Bam

On 6:40 pm
Off 9 am
37° 4:47 pm to 6:20 pm

(1) Dye
Ag MM

9 slots

Ag MM
Dye

0.8% Hx4
Ag/Seap

Yellow 90%

0.75 Recovered into 50 µl
V/cm as

L'127 BMHC
Frag⁺ hr GN (6)

Blue
Bam (iv)
BMHC PRO / PURO

Bam

Sat July 7th

Bam frequent PURO

Can't spare 12 pe

Red

- Take 12 pe L'115 BMHC. PURO mini (17)
- 12 pe 10x RI salts
- 95 pe ddw
- 1 pe (20u) Bam HI hot 82 (20u/pe)

L'127
(17) Bam

37° 2:15 to 3:30 pm
1:30 pm

(2)

Dye
W

4 slots
Ag M
Dye
Dilute

0.8% Ag/Seap
4xH

1 V/cm

Recovered into 50 µl
0.001M EDTA

L'127 (17) Bam

Blue #1 1/3 off Red

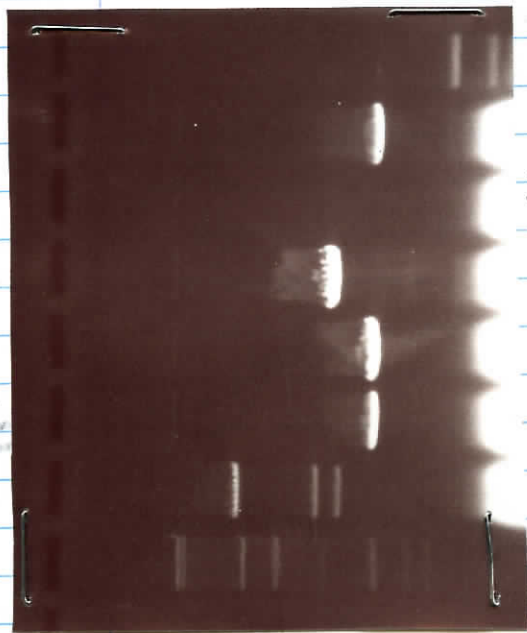
L'127 (17) Bam
Frag⁺ BMHC
PRO

① L'126 gel

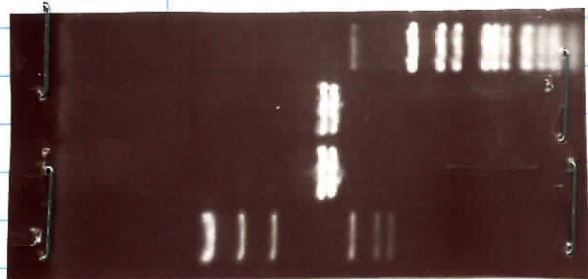


Pre
Post Bam
Post CIAP/CIAA

Bluescript

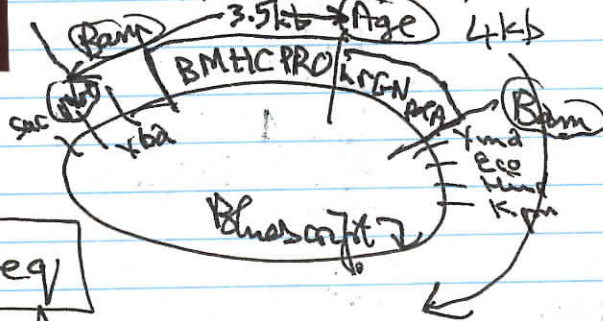


② #1, #4 & #5 look like BS ~~short~~ ~~short~~. Quite unexpected to get back #6 at L'129 ~~100~~ ⑥
The ~~other~~ ~~are~~ ~~all~~ ~~too~~ ~~small~~
#3 ~~gives~~ the vector plus ~~does not~~ the expected 4.5kb ~~band~~
~~6.2kb~~ ~~2.1kb~~ Detransform.



③ T.H. retransformed
got one candidate (#10)
Tested with Age I & Not I.

Orientation:



In computer

as blue6 bam.seq

L'129 BGN Bam
#10

②
2.1
3.5
4.5
6.2
0.8

Age
Wgt
AOR

①
②
③
④
⑤
⑥

W

W

L'129
minis
liger
Bam
12:20 PM
B
Tak
B

Sat. July 7th

Subcloning ~~BMHC~~ BMHC. hr G⁻N⁺ shot

Take 1 μ l (? 1 μ g) K-64 (Sajo) BS-II-XSt⁺
 $\checkmark$ 10 μ l 10xRI salts
 $\checkmark$ 89 μ l L¹29
 $\leq \frac{1}{2}$ μ l (10 μ) Bam HI lot 82 (20 μ l/ μ e)
 37° 4:30^m to 5:30^m + 21 μ l 10M Tris pH 8 (5x)
 + 1 μ l lab CIAA

16 μ e
 L¹29
 Pre

Bluescript
 Bam digest

1x ϕ 2x CIAA

L¹29
 BS Bam
 CIAA

White

16 μ e

L¹29
 Post

Bam Bam
 BMHC hr G⁻N⁺

Shot
 Bam

Sun. July 8th

Take 15 μ l L¹27 Blue 6
 $\checkmark$ 5 μ l L¹29 BS Bam CIAA
 2 μ l NH₄Ac 8M
 55 μ l EtOH. Freez. Cent.

L¹29
 L¹GN

Back up into 5 μ l 1x p15 ~~and~~ 1/5 μ l L¹29 T4 ligase conc
 15° 5 μ l to 6:07 μ m, RT 6:07 to 6:43 μ m, 37° 6:43 to 7:20 μ m

From 7+1+1

cds

Transformation

DK52	80 μ e $\checkmark$
L ¹ 29 lig	2 μ l $\checkmark$
0° 7:24 μ m to 8 μ m	
42° 90 sec.	

Plate 1xAMP NZY
 + Xgal 75 μ l/plate

R 20 5 10 5
 (10) (4) (16) (16)
 37° 8:09 μ m

Friday

Plate pre-spacer with
 75 μ l lab standard X-gal

5 are white

#3, #5 & #6 are clear on
 morning inspection.

L¹29 #10
 BGN Bam

L¹29 1 μ e
 minis 1-6
 digested with 20 μ l
 Bam HI (L¹27 protocol)
 12:00 μ m to 1:50 μ m
 Bulk prep

Take 6 μ l L¹29 mini
 picked another
 batch after retransforming
 correct is

2.2.2.2.
 2.2.2.2.
 2.2.2.2.

0.8 μ l 1x4
 PAX

eye
 w/pe
 AOR

1
 2
 3
 4
 5
 6

2.2.2.2.
 w

eye

130

2494

Class Name: L'130 Age Bsp E1

TTA	AGG	TTC	CGG	AAC
16				
50%	C			

242 at
N1.

(XIV)

~~Age II~~

BspEI

Man. July 9th

YN pA for BMHC YN assembly

still missing ~~one~~ piece (XIV)

Take 3 μ l (1.5 μ g) pETFP-Nuc K'119
6 μ l NEB buffer 2 10X
6 μ l ~~ATL~~ 10X
4.5 μ l ddw etc.
Don't need this - already done L'95 Age II YN

ligation:

Take 3 μ l L'95 Age II YN

✓ + 4 μ l L'130 Age II BspEI ~~Age II~~ oligo

✓ + 2 μ l p95 10X ligation buffer

✓ + 1/2 μ l L'99 T4 ligase conc.

L'131
L'13N

whed
15⁰⁰ 9:22am

to 1:45pm

RT 1:45pm

to 2pm

32⁰⁰ 2pm to T.H. for

Transformation

KAN

DH5a

80 μ l

L'131 lig

3 μ l

1X KAN, NZY

0⁰⁰

R, 20, 5, 20, 5

42⁰⁰

90sec

32⁰⁰

Error

Age of BspEI will interchange.
Re-think.

182) seq check: 7703 from: 46 to: 880
/afs/isis.unc.edu/home/n/o/nobuyo/oliver/bMHCpre.seq check: 7960 from: 1
o: 3545
Gaps: 12 Quality: 6992 Ratio: 8.621 Score: 638 Width: 24 Limits: +/-25

46 CTGCAGTCTGGATCCCTGATGTTGGTGTTCCTGGGATAAAAACTCCCA 95
 |||
 1 CTGCAGTCTGGATCCCTGATGTTGGTGTTCCTGGGATAAAAACTCCCA 50
 |||
 96 ATGTGAAGTCTCTTGCCTGCTGATGTGTGCTAGGCCCTCACCTTGAAT 145
 |||
 51 ATGTGAAGTCTCTTGCCTGCTGATGTGTGCTAGGCCCTCACCTTGAAT 100

182. Seq

183. seg *Reverse of actual* check: 9381 from: 1 to: 989
/afs/isis.unc.edu/home/n/o/nobuyo/oliver/BMHC.PURO check: 3644 from: 8174 to:
17564

ASSEMBLE July 5, 2001 10:13
Gaps: 9 Quality: 4738 Ratio: 6.153 Score: 467 Width: 23 Limits: 47-24

183. seq ← Reverse

Good fit to 3' of PURO
 ATCTTATCATGTATCTGGNGGCTGTGTCG
 960 + - - - - - + - - - - - 989
 TAGGAATAGTACATGACNCCCGGACATGGC
 Match is good considering sequence
 ANR. Real primer
 CTGGATCCTGTTCG
 Bam HI ← can be seen under the high GC

310 AAAGNAAAGCCNGGNGNGGTTTNTGNA¹NCGANCNGTNNNGGNGTNC²T 389
 8476 TGGCGCGCACCGGCCAAGGAGCCCG³.CGTGTTCTCTGGCCACCGTCG⁴ 8523
 390 CNGNNNCNANCNATCAAGGGGCAANGGGTATGGGGCAGCGTCAGTCGTG⁵ 439
 8524 GCGTCTCGCCGACCACCGAGGGCAAGGT⁶.CTGGGCAGCG⁷.CCGTCGTG⁸ 8570
 440 GCTCNCNNGGAGTGNANNNNGNCCTTAGCGCGNCGGGGTGNC⁹CGCCTTCN¹⁰ 489

PURO
→ good below

Reverse of actual Line (6)
 185. seg check: 5968 from: 1 to: 893
 /afs/isis.unc.edu/home/n/o/nobuyo/oliver/hrGNCta) seq check: 1233 from: 9920 t
 o: 19262

ASSEMBLE May 7, 2001 13:14
Gaps: 17 Quality: 6324 Ratio: 7.449 Score:

3 GTNCNATGGGCGNGGCAGAAATTTCTTGNTAACI
|| ||| ||| | ||| ||||| | |||
9951 GTGCTATGCGGCCCGCGAG.AATTCTCGTAAAAAC
NSE I
53 AGTTANAANTNCCNNNNNNCNCTNTTTTCCTTTTTT
|||| | | | ||| ||| |||||
9998 AGTTATTATTCTCTCTCTCTCTCTTTTTCTTTTTT
EcoRI
103 TTNTTCTGTGCACAGGNTNGAGTACAGGGMMCA
|| |||| | || ||||| |||
10047 TTTCTGTGTGCCAGGCTGAAGTCAGTGTAC.
NotI
153 CTNCAGNCTGGAACCTNCTGGGNTCAAGGCCATT
|| ||| |||| | ||| |||||
10093 CTGCAGCCTGGACT..CTGGGCTAAG.....
XbaI
203 GCCTGCCCCAAGTAGNTGGGNCNAGAGGGCAACA
||||| ||||| ||||| |||||
10134 CAGCTCTCCAAGTAGCTGGG.GCTACAGG.CACA

253 CAGGATAATTTTTTCATTATTTGTAGAAGAGNGAGTCTCACCATGTTACC 302

184.seq check: 6835 from: 46 to: 917
/afs/isis.bnc.edu/home/n/o/nobuyo/oliver/BMHCpro.seq check: 7960
o: 3545

Gaps: 10 Quality: 5700 Ratio: 6.746 Score: 506 Width: 7 Limits: +/- 8

46 CTGCAGTCTGGATCCTCGATGTTGGTGTTCCTGGGATAAAAACTTCCA 95
1 CTGCAGTCTGGATCCTCGATGTTGGTGTTCCTGGGATAAAAACTTCCA 50
96 ATGTGAAGTCTCTTGCCTGCTGATGTGTGCTAGGCCCTCACCTTGAAT 145
51 ATGTGAAGTCTCTTGCCTGCTGATGTGTGCTAGGCCCTCACCTTGAAT 100

184. seq.

①

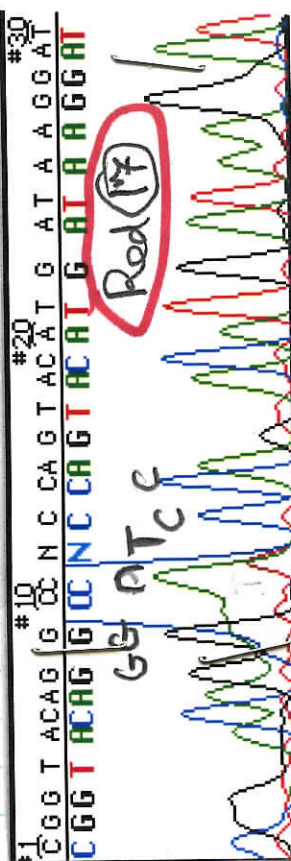
EcoRI mluI EcoRI XbaI Kpn

TTGATACCGGAATTCAACGGTGAATCTAGACCTCTANGTACCGGT

1

AACATATGGGCTTAAGTCGGCACTTAAGATCTGGNGATNCATGGCT

④5



Rad (5) BMHC: PUAO

Red 17

Recd (17)
BMHC. PURO

Blue

Blue Check out
OK at this time

Tues. July 10th

Sequencing results

Red (5) BMHC PURO

L' 115 (15) Blue Mlu L' 17 (seigo 1)
Begins fine Eco Mlu Eco Aat II → BMHC PRO

180. seq

185. seq

OK

OK

L' 115 (15) Red Aat L' 121 (seigo 4)
From 17 it runs 5' to 3' in BMHC PURO
but sequence is ~~forward~~ (from Red primer) so
orientation is wrong
looks like BMHC PRO ORND

Wrong

Wrong

Red 17

L' 115 (17) Blue Mlu L' 17 (seigo 2)
Begins with Eco Mlu Eco Aat and goes into BMHC PRO perfectly

182. seq.

OK

Red 17 BMHC PURO

L' 115 (17) Red Aat L' 121 (seigo 5)
Starts correctly at L' 121 Aat and runs back into PURO fine.

183. seq.

OK

Note: This is hCGN pot A (long)!

BMHC hCGN long

Blue 6

L' 119 (6) Blue Mlu L' 17 (seigo 3)
Is correctly at 3' end of 5' HPRT homology with Eco. Mlu. Eco..... Kpn. Pst.
but only to ~1600 in BMHC because sequence is poor (?)

184. seq. XbaI

OK

Blue 6 Blackout OK at this level.

BMHC hCGN long

L' 119 (6) Red Aat L' 121 (seigo 6)
Has no BMHC PRO sequences

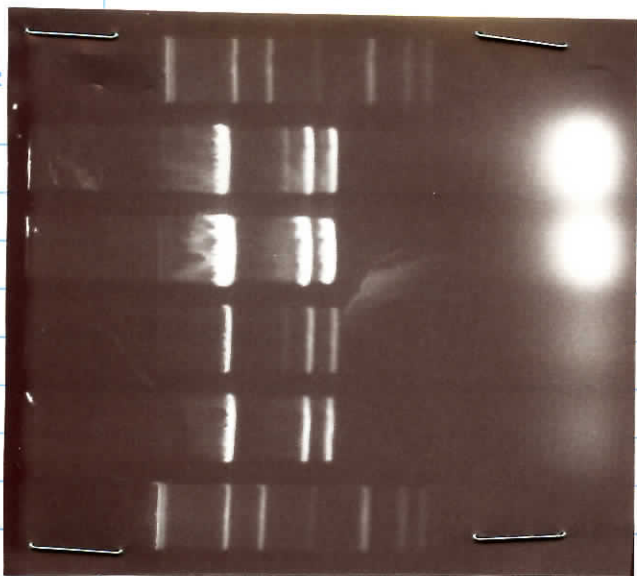
185. seq.

Clear reverse sequence of seigo 6, equivalent to 3' end of HPRT
Beginning 11nt. 3' of end of HPRT

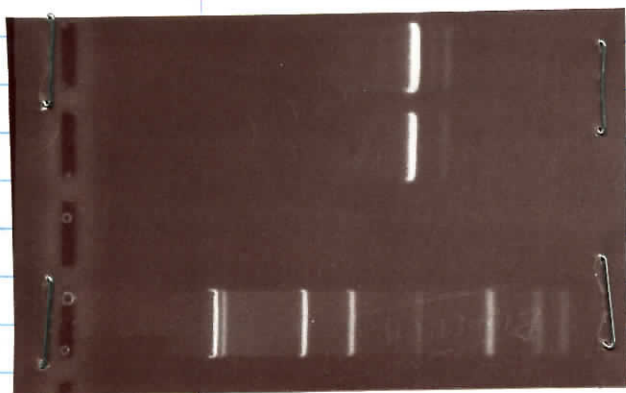
OK

Conclusion: Blue 6 & Red 17 are OK

134



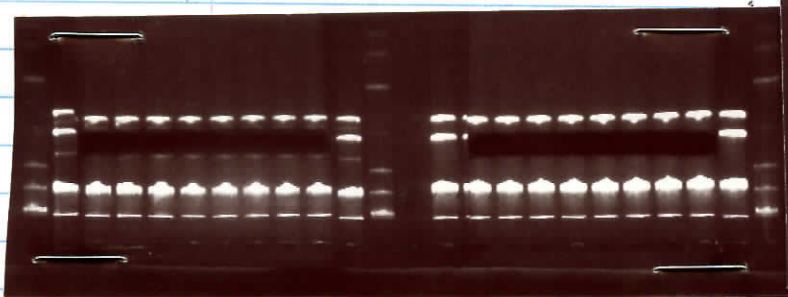
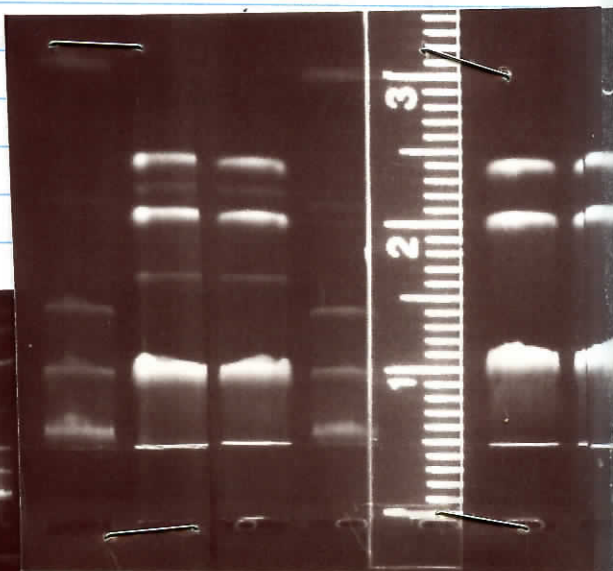
① Good cuts etc. Use Blue ⑥ undiluted. Use Red ⑦ at 70%



③ ~~Good~~ Both fragments are in a separate yield & purity.

Good cuts, but Blue ⑥ G.N. has same impurity.

②



ne (6)
at 70%

on 9:55 am
off 10:40 am
Max 0.8 Hx4

Thurs July 12th

135

(Bam) BMHC PRO. PURO of L135 (Bam)

Ag

Blue (6)

Red (17)

Blue (6)

Red (17)

Ag

W

Dye

Bam ← from T.H. for bulk prep. 40 pl mini
to 240 pl total
400 Bam 2.5m

Check of conc. prior to bulk prep.

on 9:55 am
off 10:40 am
Max 0.8 Hx4
Ag + seap. 1V/cm

G-N
L135 Blue (6)
Bam Fragt

L135 Red (17)
Bam Fragt
PURO

Ag
Hg
10 slots } Blue (6) mobilized
Ag
10 slots } Red (17) 105 + 45
Ag
Dye

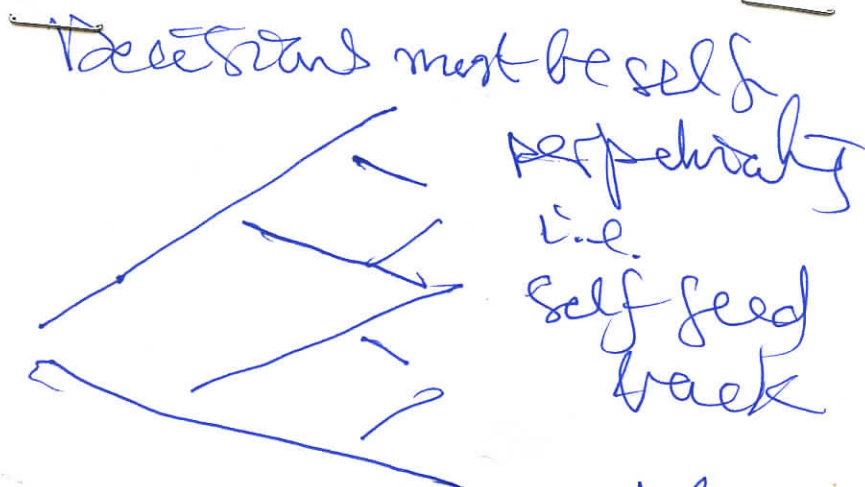
~~Blue (6) mobilized~~
~~Red (17) mobilized~~

on 9:55 am
off 11:35 am
Max 0.8 Hx4
1V/cm

Dye
W
Blue (6) fragt
Red (17) fragt
W
Ag
W
Dye

L135 Blue (6)
GN Bam Fragt

L135 PURO
Red 17 Bam Fragt



Random L vs R switches

10 choices $\rightarrow$ 1000 cell types



Thurs. July 12^d

137

Further switching program ideas

Realized two things:

i) that bifurcation choices requires very few steps to get to great diversity if the choices can be in all combinations & the order of steps means something.

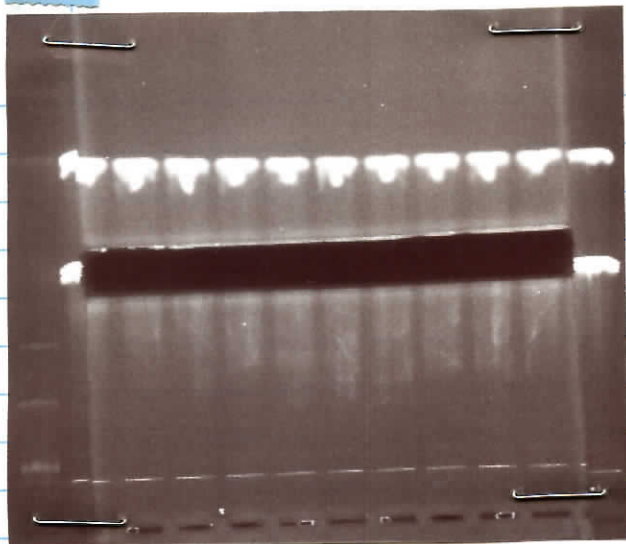
ii) that autofeedback is essential at "threshold" c.f. various bistable models of phosphorylation & dephosphorylation that have been published.

Experimentally:

i) current obtaining of cardiomyocytes from ES cells suggests only a few steps.

ii) hematopoietic stem cells to cardiomyocytes also suggests same - a few steps.

iii) may be able to make a BMHC PURO AMHC NEO animal & switch HSC to cardiomyocytes



① good cut, but not brave!



② changed drying of gel ppt to 2 min. on suspicion of too dry for proper cut.

Good yield of ^{Not} BMHC w/ ^{Eco} BN
Eco Not frag^t.
Purity acceptable.

②
0.86 HxL
on 5m
0.86

Dye
W
AG
W
L139 B
L139 B
V
10

Tak
Volume
L139
Eco Not
BGN

Red
L139
Eco
BP

Sat. July 14th

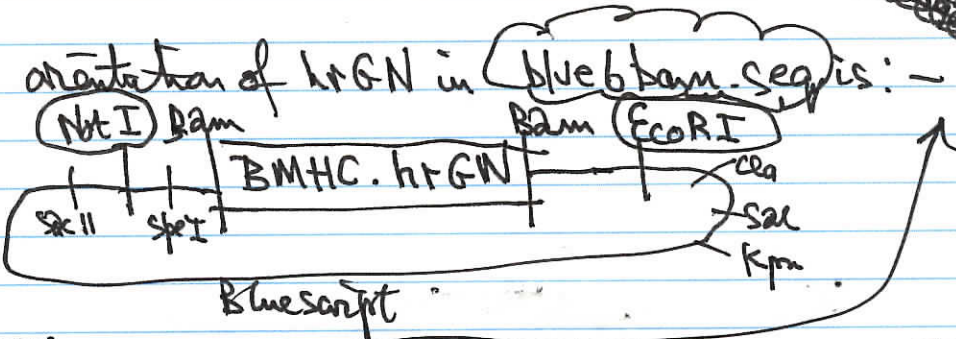
139

②
16 min
16 min

Preparation for Assembly of modified (vii)

Note

Dye
W
AGMM
W



L139 BMHC h+GN
Eco Not Frag
L139 BPURDEco
W
Dye

to do this I need

L139 Blue linker + L139 Red linker

to modify (iv) 5'-end to Eco... Not... Eco. Both orientations will occur; can distinguish by sequencing or PCR.

✓ 200 pcds W

Take ✓ 12 pL L129 BGN Bam #10

Blue
Eco Not
BGN

✓ 24 pL 10x Eco RI salts

✓ 1.2 pL (24 u) Eco RI Lot 29 (20 u/pL)

✓ 2.4 pL (24 u) Not I Lot 18 (10 u/pL)

37° 5:20 to 22:30

29536
22
45%

L139 Blue Eco Not					
AA T	T C T	T T T	G C G	G C C	
G C T	T T T	C			

29537
22
45%

L139 Red Eco Not					
AA T	T G A	AAA	G C G	G C C	
G C A	AAA	G			

Red

Take 115
✓ 40 pL L139 Red (17)

L139
Eco
BPUR

✓ 174 pL ddw

✓ 24 pL 10x Eco RI salts

✓ 2 pL (40 u) Eco RI Lot 29 (20 u/pL)

37° 5:20 to 22:30

Heat inact. ≥ 80° ≥ 10 min.

(Xviii)

L139 BMHC h+GN
Eco Not Frag

60 pL

① 0.8/10 Hx4/10 A9/10

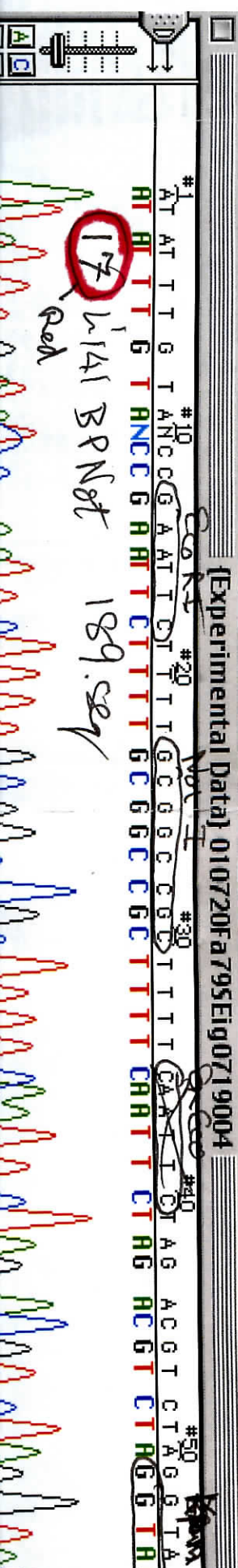
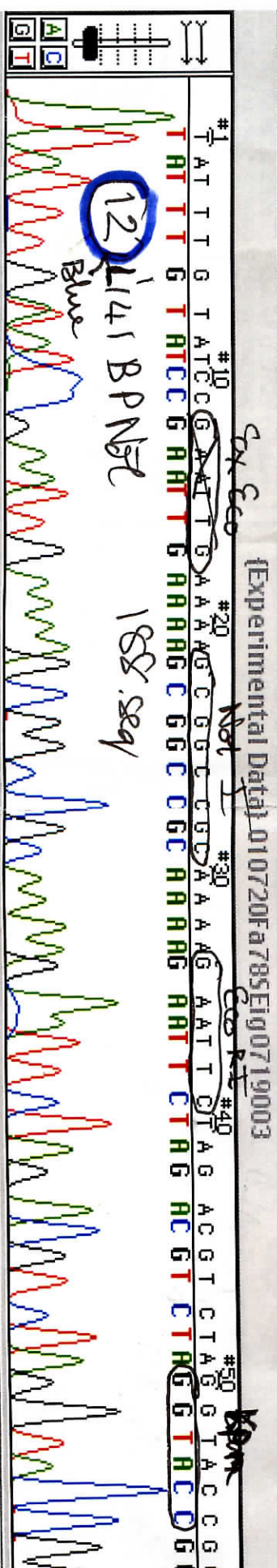
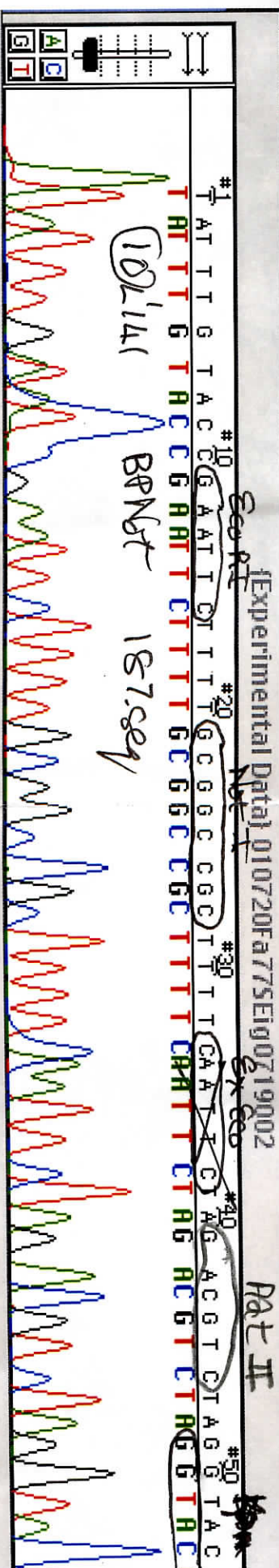
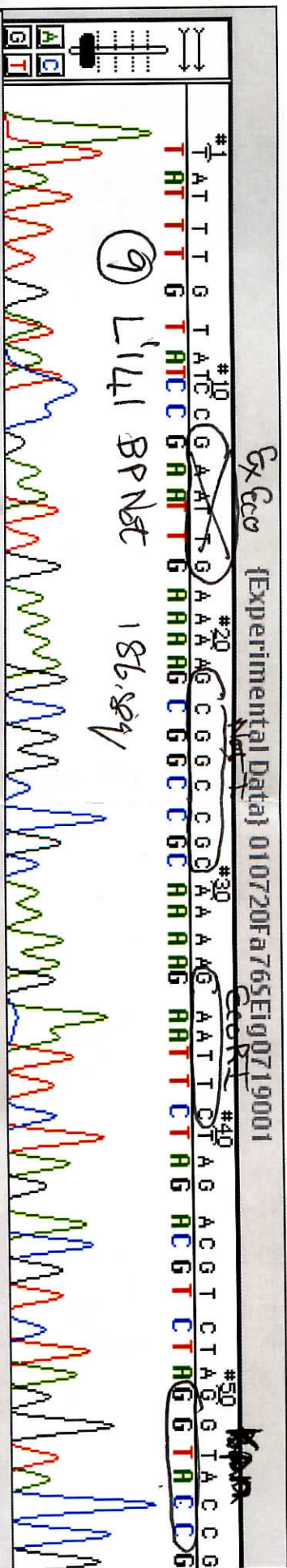
(dilute) AGMM

11 slots
L139
Eco Not
BGN

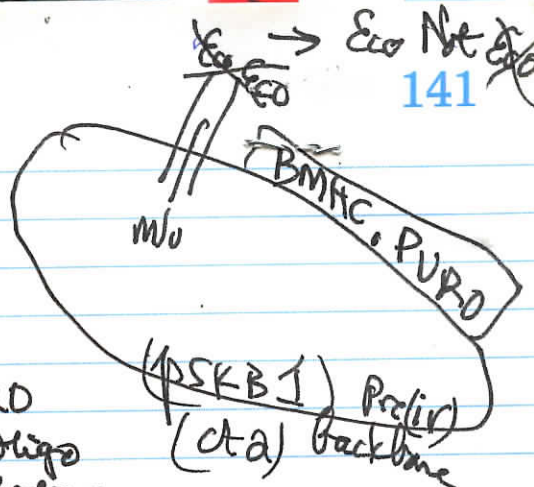
On 750 pL

2nd Blue 2/31

Mon
10:00
Eco-Not



Mon
Screen July 18th
change in
Eco-Not ~~BP~~ BMHC.PURO



L141
EcoNot
LGN

- ✓ 6 µl 2dW Red
- Take 3 µl L139 Eco BPURD
- ✓ 4 µl L139 Blue EcoNot oligo
- ✓ 4 µl L139 Red EcoNot oligo
- ✓ 2 µl 10x p95 buffer
- ✓ 1 µl 199 T4 ligase conc

15° 9:35 am to 1:25 pm, RT 1:25 pm to 2:45, 37° 2:45 pm to 3:55 pm

T.H. Subcloning Transformation

9 & 12
are Not Eco BMHC

10 & 17
are Eco Not BMHC

DH5α 80 µl
L141 EcoNot
wign
to —
90 sec 42°

(Amp)
Plated 1x Amp NZY

R, 20, 5, 20 / 16, 5 / 16 37°

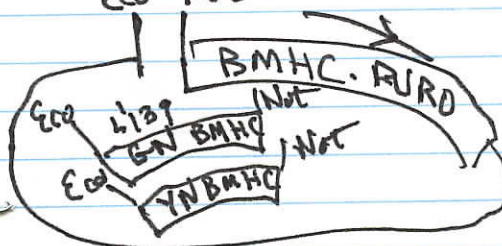
Screen with Not

T.H. test 21/24 were correct by Not I screen.
Test 9, 10, 12, 17 by K17 Mlu blue sequence.
All 24 labeled *
L141 ~~BP~~ BP Not
#1...24

Sequence 4 to get orientation using
K17 Blue Mlu primer

Then cut with Eco and Not
to receive L139 BMHC LGN Eco Not frag^t
or equivalent BMHC LN Eco Not frag^t

Continued
on L155
with Blue 12
& Red 17



510
over

Eco0109I
Psp0MI I
KpnI I I
5I I I I
I I I I

INSERT
Bonn/Eco

EXERCISE

AarI	AatII	AfeI	AflII	AgeI	AscI	AvrII	BaeI
BbeI	BbsI	BbvCI	BclI	BglII	BlpI	BplI	Bpu10I
BsaBI	BseRI	BsgI	BsiWI	BsmI	BsmBI	BspEI	BspMI
BsrGI	BstAPI	BstBI	BstEII	BstZ17I	Bsu36I	BtrI	EcoNI
FseI	HpaI	KasI	MfeI	MluI	MscI	NarI	NcoI
NdeI	NheI	NruI	NsiI	PacI	PflMI	PmeI	PmlI
Ppu10I	PpuMI	PshAI	RsrII	SanDI	SbfI	SexAI	SfiI
SfoI	SgfI	SgrAI	SnaBI	SphI	SrfI	StuI	StyI
SwaI	Tth111I	XcmI					

(2) ~~02/11/2018~~
~~02/11/2018~~
~~02/11/2018~~

Worksheet 143

25

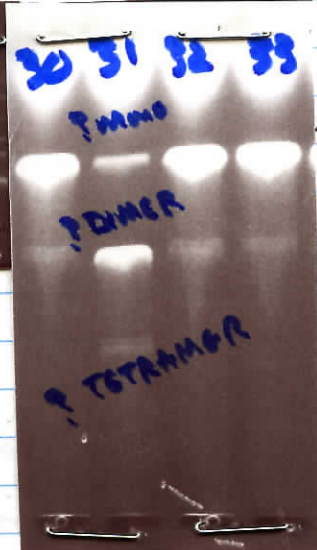
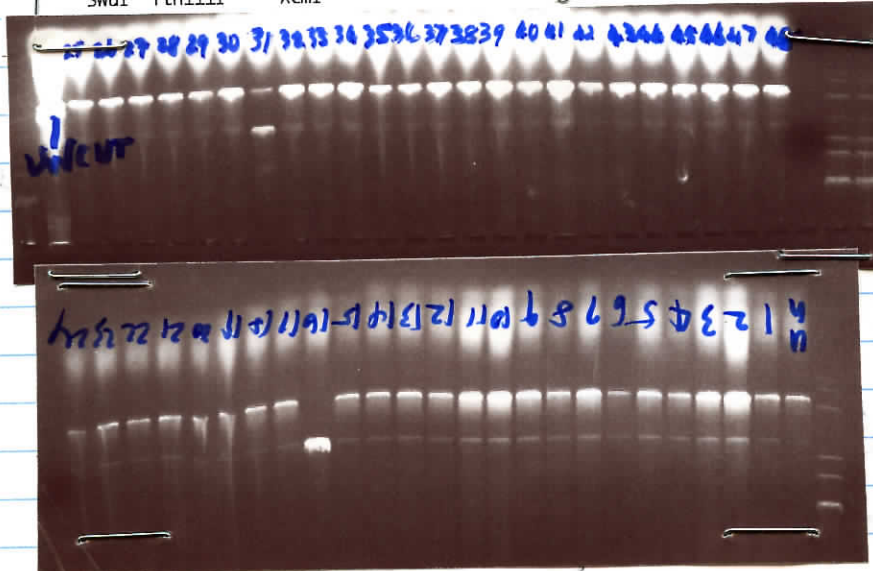
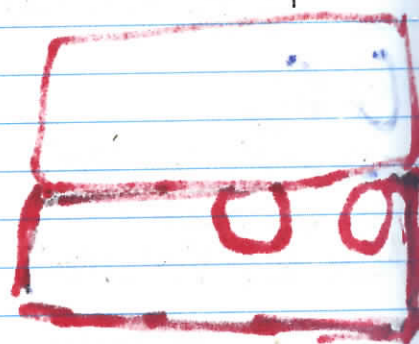
Exo (48)

Date _____

AG

AG

L'14
Eco
L16



EcoRI
ligated
forming

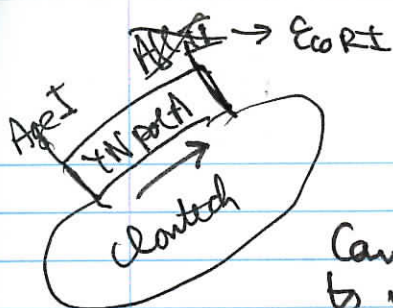
Ques (2)

3 is odd - more likely a times.

Try a new strategy. Tomoko did 24 more - all negative.

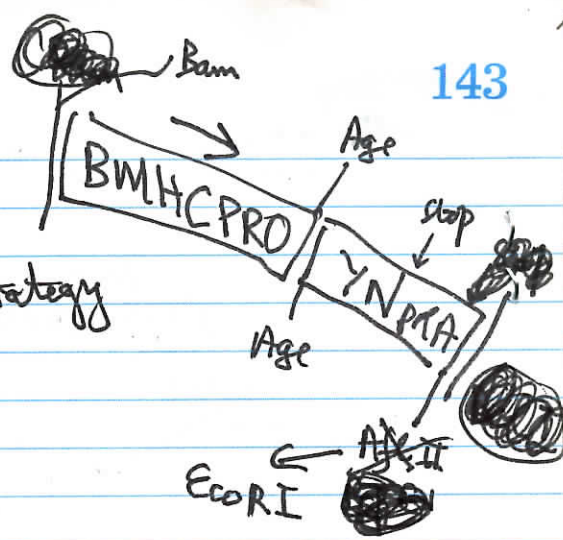
143(25) - (45)

10:55
 11:00
 11:05
 11:10
 11:15
 11:20
 11:25
 11:30
 11:35
 11:40
 11:45
 11:50
 11:55
 12:00
 12:05
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 23:35
 23:40
 23:45
 23:50
 23:55
 24:00



Tue
 Sun. July 17th

Can subclone into BS
 to use with EcoRI Not strategy



Need to change Aff II in YN
 to EcoRI (which does not
 cut YN).

11 pe ddw
 Taber 3 pe L'95 Aff II YN
 4 pe L'143 Aff II EcoRI Aff II dig
 2 pe 10x p95 lig. buffer
 1/2 pe L'99 T4 ligase

L'143	Age II	EcoRI	Age II
TTA	AGA	CGA	ATT
C			

15⁰⁰ to 2:00am
 15⁰⁰ to 2:15pm
 RT 2:15pm to 3:10pm
 37⁰⁰ 3:10pm to 4:40

To T.A. for transformation
 S.H.

Tested with Eco RI

Insert (Bam) BMHC PRO (YN) (Eco) into BS and use

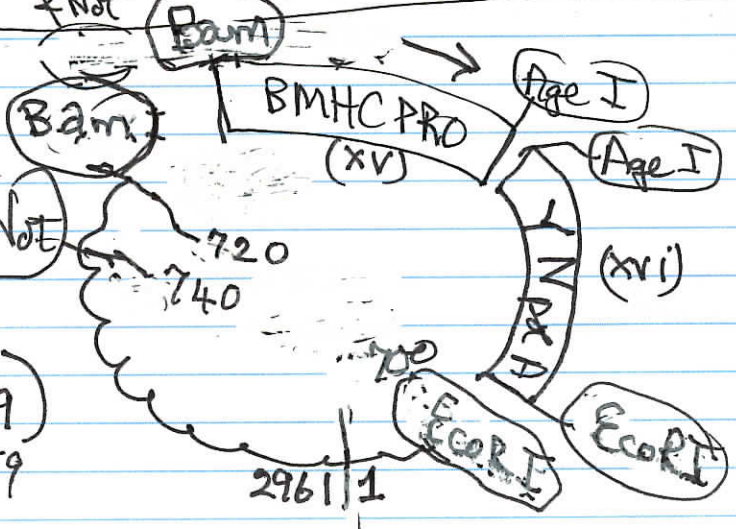
Eco RI and ~~Not~~ Not for excision

Then insert into ~~Eco~~ Not
 BMHC PRO

L'141 BP Not
 actual Eco
 & Not

Thurs July 19th

Three piece assembly
 prior to excision
 with Not I & Eco RI
 as (XVII)



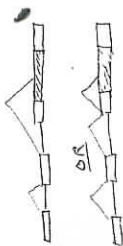
(Continued on L'149)
 on L'159

-all

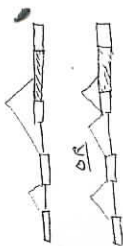
Step 1) Insertion of AMHC cDNA

We have used the α -MHC/Hyg construct to express three different myosin light chains. When we expressed the MLC2V and MLC2F cDNAs, we found that full length, functional light chain proteins were made. When we expressed the MLC2A cDNA, no protein was made. I checked the RNA by RT-PCR and found that the construct had spliced incorrectly.

Expected splicing



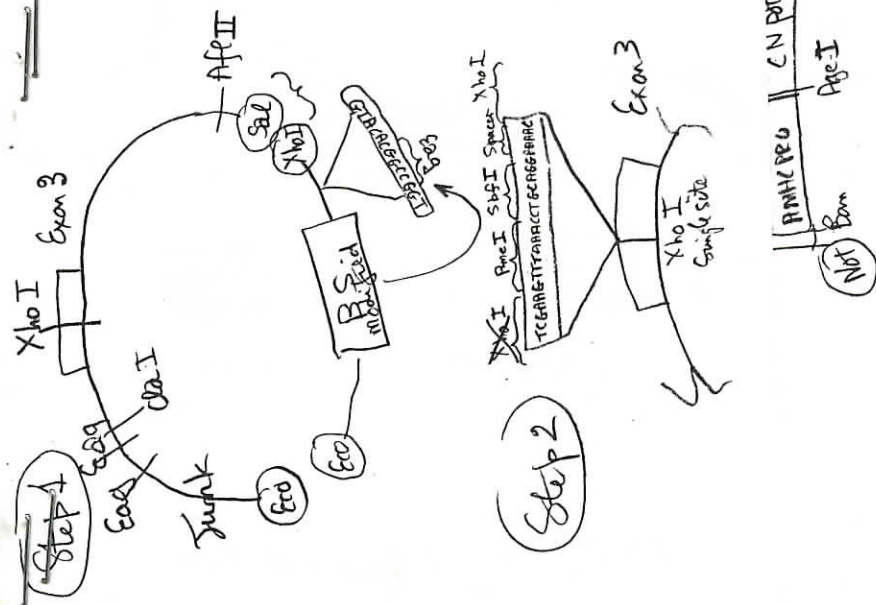
Observed splicing



Good luck
Jim Gulick

AMHC better

L145



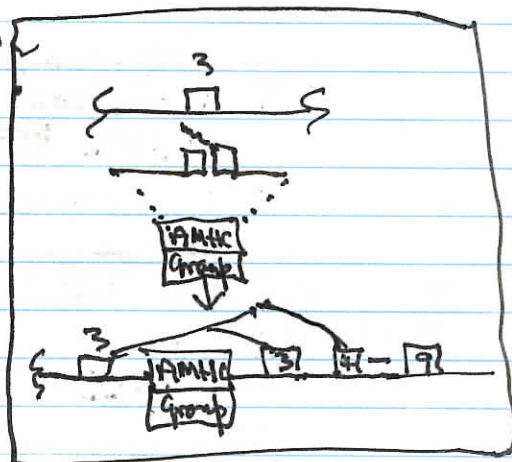
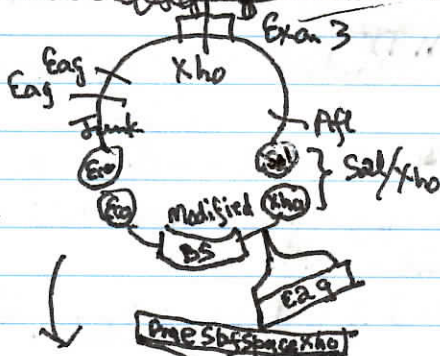
splicing across the group AMHC

Wed. July 18th

AMHC.NEO/AMC.CN

Following several step scheme assembled in computer as 7.16.inter 1 7.17.inter 6. Aim is to take BMHC set (HPRT⁺) and go to (BMHCset)(AMHCset)(HPRT⁻).

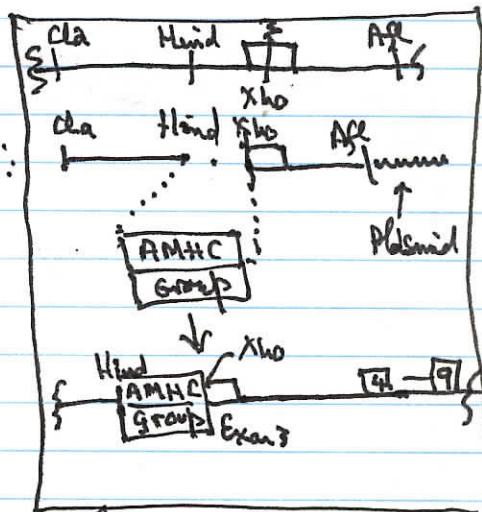
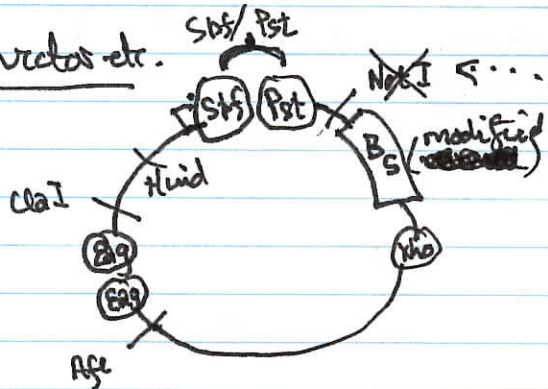
Step (i) Isolating ~~several~~ 3' homology



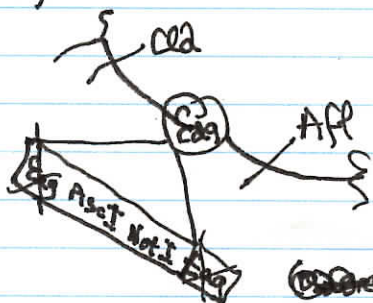
Step (ii) modifying Exon 3

Xho

Step (iii) Reassemble vector etc.



Step (iv) Modification



Conclusion

Consider going to Σ (not $\subset$)
or using a different strategy (N.M.)

Steps

- L'157 (i) subclone 6 kb ApaI frag⁺ from h⁺ 6Nct2. seg into BS
 isolate (a) 5' homol. 4.1 kb Apa - Hind and 3' homol Xho - Afl 1.2 kb
 (b)
- (ii) pMC1 Neope A ~~cloning~~ single site Pst → Sal M'9
 M'3: Bam HI → Hind III. Isolate (c) Sal - Hind 860 bp
- (iii) Isolate (d) Sal - Not from AMHC. 5.5 kb
- (iv) change Sal → Age I in AMHC & isolate (e) Not - Age 5.5 kb
 M'13
- * (v) change Bsi WI = M'11: in clontech CN pcr A to Xho → isolate (f) Age - Xho 1 kb
 using BS
- (vi) Assemble (c) + (d) into Hind - Not I & (e) + (f) into Not -
 in BS and isolate (g) Hind - Xho 13 kb and reassemble Xho
- (vii) BS change Hind III to Afl II & isolate (h) Apa → Afl 3 kb
 M'11
- (viii) 4 piece assembly (a) (g) (b) (h).

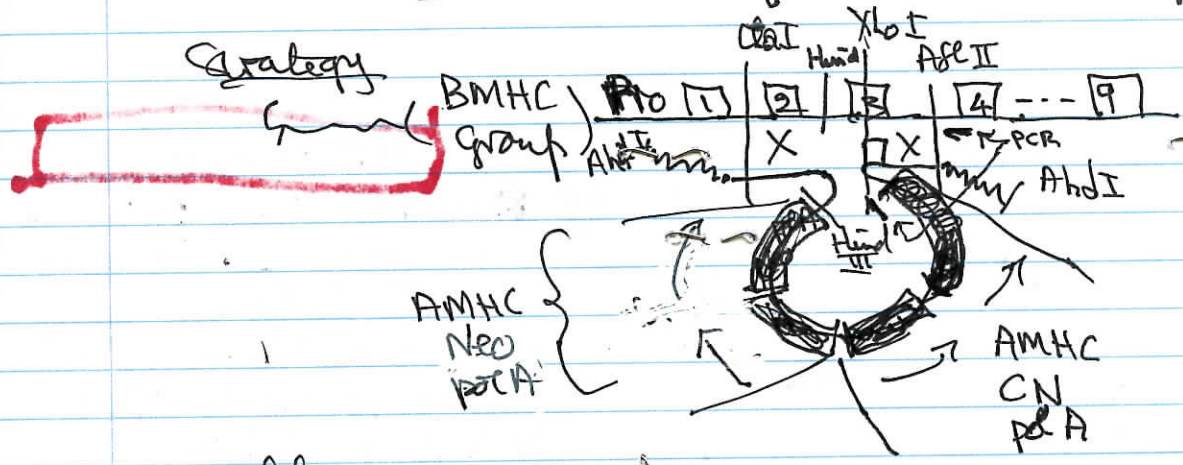
Final product can be used on group B HPRT⁺ cells
 or on wild type cells.

BS
1.2 kb
1'9
1 kb
Xho

Thurs July 19th

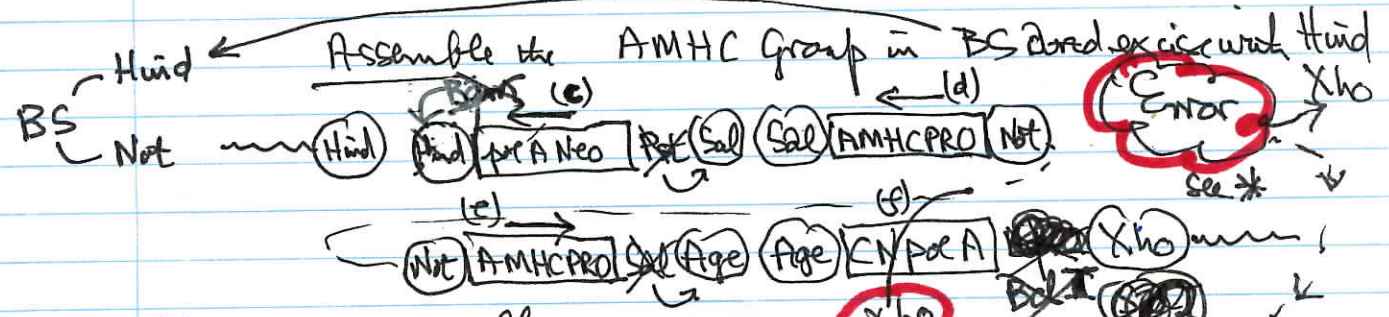
NAA C omega for second group

Strategy

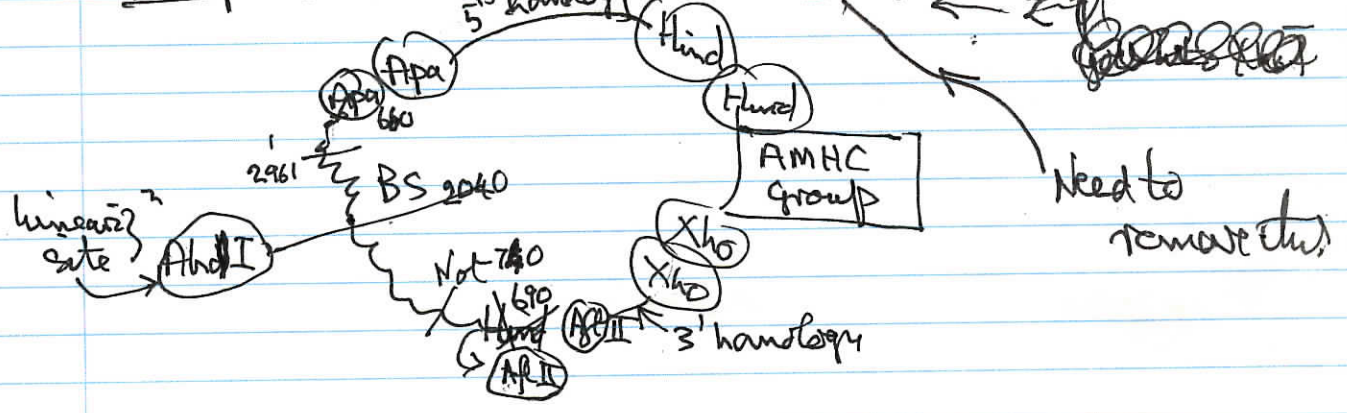


Assembly

L157 (a) Subclone 6 kb Apa I from hG NctA seq into BS.
 (b) Isolate Apa I 4 kb Hind III & Xho I 1.2 Age II
 5' homolog, 3' homolog.



Four piece assembly





① Escape RNase! Cut for BMHC is good; for YN is prob. OK. - but see below ↘

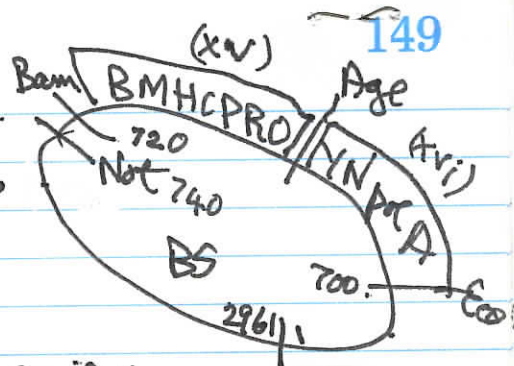


The L149 BMHC Age/Bam is of
 ② Reasonable purity; the 3 kb band is not ~~one~~ one type cut ~~thru~~ ^{thru} script. Something wrong with YN pol A; it sh. be 1070 bp + 6 linker; it is 740bp. Compare Age YN pol A Age Spe on L106/1/95

①
 Invi by
 Recover

Thurs July 19th

3 frag^s ^{their} assembly ~~into~~ →



Take ✓ 88 µl ddw

✓ 10 µl 10x RI salts

(w/149) ✓ 1 µl Bluescript BSI KSC(+) Kdt from S.H.

✓ 1/2 µl (100) Bam HI lot 82 (20 u/µl)

w/1 ✓ 1/2 µl (100) Eco RI lot 29 (20 u/µl)

L'149
BS
Eco/Bam

Blue

37° 2^{pm} to 4¹⁵ inact, ≥ 20 min ≥ 80°C

Take ✓ 85 µl ddw

✓ 12 µl 10x RI salts

✓ 12 µl L'101 BMHC PRO mini (16) (i)

✓ 1 µl (20 u) ~~lot 82~~ lot 82 (20 u/µl) Bam HI

✓ 10 µl (20 u) Age I lot 33 (2 u/µl)

L'149
BMHC
Age/Bam

Red

37° 2^{pm} to 4²⁰ to cold

Take ✓ 85 µl ddw

White

✓ 12 µl 10x RI salts

✓ 12 µl L'143 YN (16) EcoR TH 4-28

✓ 1 µl (20 u) lot (20 u/µl) Eco RI

✓ 10 µl (20 u) Age I lot 33 (2 u/µl)

L'149
YN
Age/Eco

37° 4^{pm} to 5:30^{pm}

w/1.2^{pm} HX 2.5
max 12:10^{pm}
at 12:40^{pm}

On 6^{pm} 9 am HX 4/A9
off 0.5^{pm} 8^{pm} 1^{pm}/am

Age
AG MM

8 slots BMHC red Age/Bam (3.5 kb)

L'149 BMHC (xv)
Age/Bam frag^t

1st blue 90%

Age
AG MM

9 slots YN white Age/Eco (1 kb)

L'149 YN
Age Eco frag^t

white

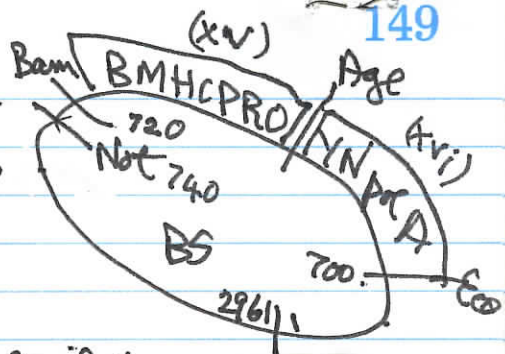
Continued
L'151

Age lot
AG BMHC
L'149 Age Bam
L'149 YN Age Eco
PCR w/
dye

Immun
Recap

Thurs July 19th

3 frag. ^{their} assembly ~~into~~ →



Take ✓ 88 µl ddw

✓ 10 µl 10x RI salts

(w/ps) ✓ 1 µl Bluescript BSII KSC(+) K44 from S.H.

✓ 1/2 µl (10u) Bam HI hot-82 (20u/µl)

w/pe ✓ 1/2 µl (10u) Eco RI hot-29 (20u/µl)

L149
BS
Eco/Bam

Blue

37° 2pm to 4:15pm inact, ≥20min ≥80°C

Take ✓ 85 µl ddw

✓ 12 µl 10x RI salts

✓ 12 µl L101 BMHC PRO mini (16) (i)

✓ 1 µl (20u) ~~BSII~~ hot-82 (20u/µl) Bam HI

✓ 10 µl (20u) Age I hot-33 (2u/µl)

L149
BMHC
Age/Bam

Red

37° 2:20pm to 4:20pm to cold

Take ✓ 85 µl ddw

White

✓ 12 µl 10x RI salts

✓ 12 µl L143 YN (16) EcoR TH4-28

✓ 1 µl (20u) hot (20u/µl) Eco RI

✓ 10 µl (20u) Age I hot-33 (2u/µl)

37° 4pm to 5:30pm

L149
YN
Age/Eco

w/1.2g Hx2.5
max 1.1g 10pm
90% 12:40pm

Age hot
AG
BMHC
L149 Age Bam
L149 YN Age Eco
PCR
dye

①
Imm in
Recovered into
60µl

Age
AGMM

8 slots BMHC red Age/Bam (3.5kb)

L149 BMHC (xv)
Age/Bam fragt

Red

1st blue 90%

Dye
AGMM

9 slots YN white Age/Eco (1kb)

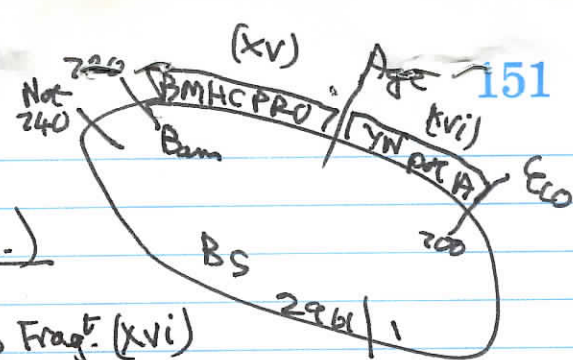
Dye

L149 YN
Age Eco fragt

White

Continued
L151

Fri. July 20th
 Assembly (contd.)



* Take 30 μ l L'149 YN Age Eco Frag⁺ (xvi)
 Hold, pending better (xvi).

(1) Dye
 T.H. will digest YN seq (Clontech K'119) with
 K'119 Age Age I + Eco RI, Age I + Age II, and (bulk) Age II. Can
 K'119 Age Age II. Purify the Age II frag⁺ & re-ligate with oligo.
 K'119 Age Age II. T.H. set up at 3 pm. Off at 4:40 pm. Gel on at 5 pm
 7 slots (Clontech & NA)
 T.H. (bulk) 0.8% Hx4 Seap. Agar. 1V/cm
 Dye
 2nd blue 40%
 Set July 21st
 Freeze frag⁺ (+NH₄Ac) pending
 isolated as

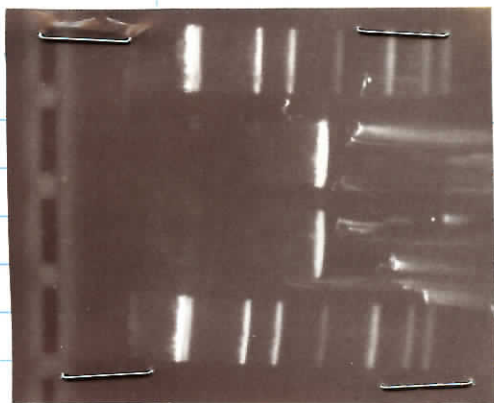
Sample cut Age II YN seq Clontech
 Error: this is only present at one site

(2) 2:50 pm
 On 4:20 pm
 Take 35 μ l 10x RI buffer
 303 μ l ddw
 12 μ l (6 μ g) YN seq (Clontech K'119) one site
 Warm to 37°
 + 1.2 μ l (12u) Age II lot 33 (10u/ μ l) 1997
 60 μ l
 "0", 1, 2, 4, 8, 16 Rest
 80° $\geq$ 10 min

Recovered 206 μ l. Ppt^d & saved dry
 L'151 YN (16)
 L'151 YN Recovery Age
 Red

0.8% Hx4 Max $\rightarrow$ to 1/3 at 1 pm. Do not expect to see the 400 bp
 in this gel, but large ch'd change.

1



① All is okay; the sizes match. Go ahead with back up of strips & purified YN. 89 ACR

2-35
only 1.60

② Dye
Ag
Ac

①

②4

Ag
W
Dye

③

W
ACR

①

②

③

④

⑤

⑥

⑦

⑧

⑨

⑩

⑪

⑫

⑬

⑭

⑮

⑯

⑰

⑱

⑲

② Name of L'153 was correct. Start again with purified Aff II fragments

③ Poor digestion - but all are correct. Proceed.

③

← Use this #13

L'153 ligⁿ

~

⑲

ACR

Ag

Dye

⑳

㉑

㉒

㉓

㉔

㉕

㉖

L'143 ligⁿ

Mon. July 25-80

L'153 Transformation

DH5α
* L'153 YNE
high

80 μl

3 pl

to

90 sec

37° 6pm
Kan

NZY

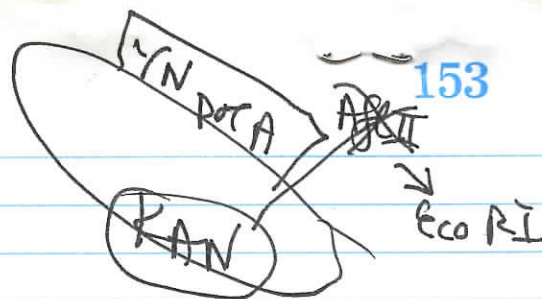
R. 20, 5, 20, 10, 5/16

t.H. did this

ahead
 1.8% Agar
 1.6% H₂O
 2N/Am

Sat. July 21

Rock-ups



L'153 DH5 α 10/1 80 μ l
 L'143 YN Eco lig 3 μ l

07:02^{pm} to 7:25^{pm}

KAN

L'153 minis
 ①-②④
 1x Kan NZY
 37° 4:30^{pm}

90 sec 42°
 Around

1x Kan NZY

37° 7:35^{pm}
 1^{pm}

R, 20, 5, 29, 5
 ②② ②①② ⑤ ① ① ① ①

Sun. July 22nd

Age cut check

L'153
 YN Age

Take ✓ 4 μ l 10x RI salts

✓ 34 μ l ddw

✓ 1 μ l K'119 clonase YN seq

✓ 1 μ l Age I (20) lot 33 (20 μ l/pe)

37° 9:50^{am} to 12:20^{pm}

①
 Age
 w
 Ag
 L'153 YN Age
 3+12 L'151 YN seq
 Ag
 w
 Age

(*)

Take ✓ 5 μ l L'151 YN seq AffII (isolated frag?)

✓ 8 μ l ddw

✓ 2 μ l 10x p95 lig- buffer

✓ 4 μ l L'143 AffII Eco RI AffII oligo

✓ 1 μ l L'99 T4 ligase

Sunday Oct 4-15^{pm}

L'153
 YN/Eco
 lig-

Mon. July 23rd

Get above

Screening L'153 minis ①-②④. Use Age I and Eco RI with 1.6% Agarose 2x Hgel.

Take ✓ 100 μ l 10x RI buffer

✓ 845 μ l ddw

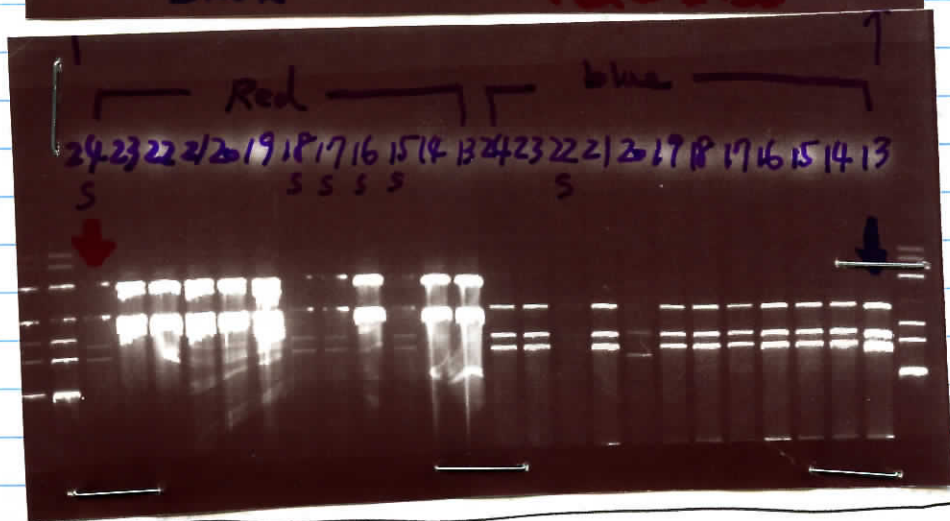
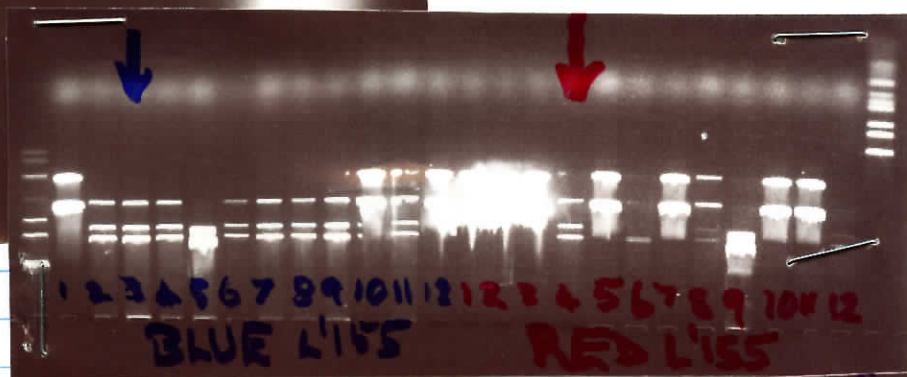
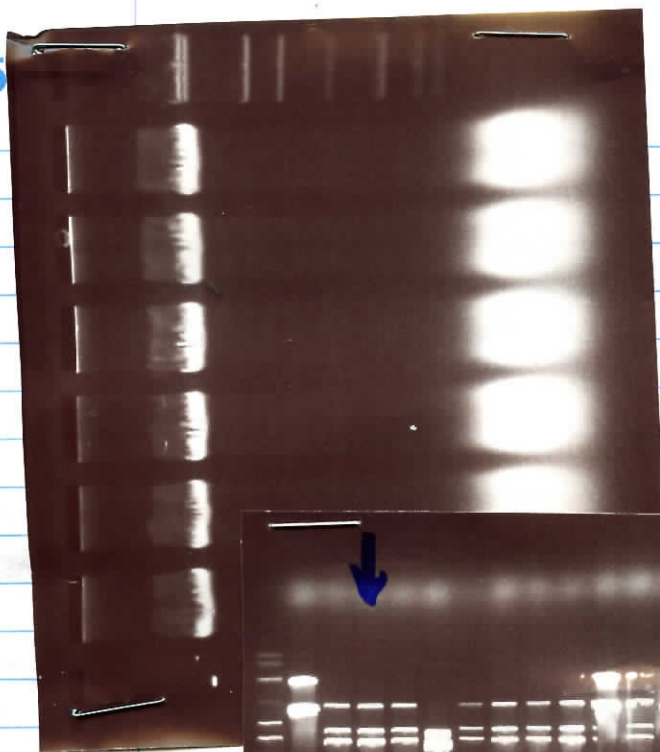
✓ 5 μ l (100v) Eco RI lot 29 (20v/ μ l)

✓ 50 μ l (100v) Age lot 33 (20v/ μ l)

L'153
 4 μ l minis ①-②④
 40 μ l aliquots

37° 2:00^{pm} to 3:10^{pm}
 3:35^{pm}

① Enzyme digestions
are fine.



Only 30
Dye
W
AG
①
Blue 12
Spe
①
Red 17
Spe
①
PCR
W
Dye

T.H. made 12 Blue (12) & 12 Red (12) minis. Check w/ Eco Spe
- expect ~9kb & ~14kb. Parent will be 4.5kb & 14kb.
Re-evaluation of sites shows expected 10kb, 5.5kb & 7.6kb
Parents will be like 20 Blue & 22 Red.

Using K'17 Minis will sequence 4 & 24 Red and 3 and 13 Blue

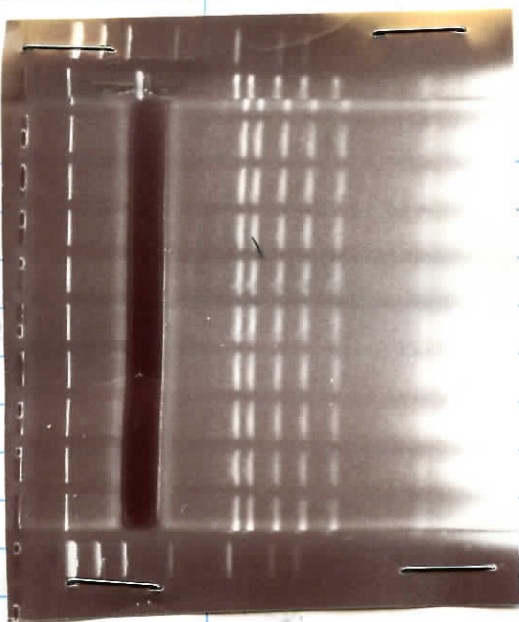
Using L125 Red Avr will sequence 4 Red & 3 Blue.

Continued M' 4 & 5

Transformation

Blue (12)	Red (17)
10/11 DH5 α L'153 ~ 90 μ l ~ 90 μ l	
L'155 (12) $\sim$ high 3 μ l L'155 (17) $\sim$ high 3 μ l	
0°C 5:14 to 5:35	37°C 5:52 pm
90 sec 42°C	
Plated 1X Amp NZY	
Blue (12) L'155	Red (17) L'155
R, 20, 5, 20/16, 5/16	R, 20, 5, 20/16, 5/16
Mixed size colonies	Mixed size colonies
This will be	This will be
	
GB, BP Blue L'155	GB, BP Red L'155

Expected to be better



① Conclusion: Cuts ok
but Pvu was incomplete.
Since the 5 kb contaminant
includes Bluescript, it
would be wise to
re-cut with Pvu prior
to subcloning, or be
prepared to screen
many.

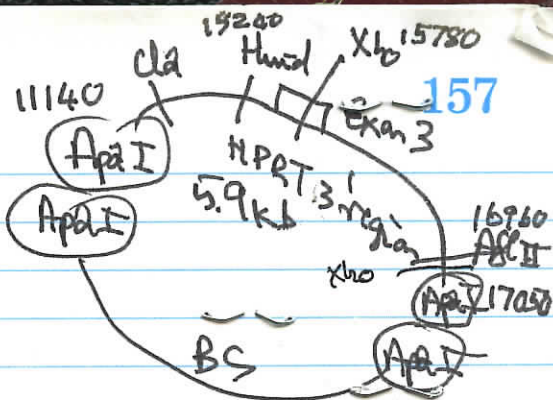
(F1)

L157
HPRTA⁺ RS

Transformation	DH5 23	1 T.H. 6/8	80 µl
Plated 1x AMP NZY (75 µl x gal. earlier)	L157 high ⁿ	0 th 6:05 pm	3 µl
R, 20, 5, 20, 5/ 16, 16 37°	6:45 pm Wed	42° 90 sec	
many - (100 µl) (14) W	T.H. make mind 2 8 minutes. All well correct.		

Tues. July 24th

Start of NAAC Omega



L'157
BS Apa
CIAA

Take ✓ 2 µl Bluescript^{L'149}

✓ 21 µl ddw

✓ 3 µl 10X NEN buffer 4

✓ 3 µl 10X Alb.

✓ 1 µl (100) Apa I hot 30 (100/µl)

Red 37° 2:20 pm to 3:20 pm

plus ✓ 7.5 µl 5X CIAA buffer

✓ 1 µl (100) CIAA lab stock

55° 3:27 pm to 3:57 pm

1X PCH/HOQ 2X CIAA

Pvu I at 500 and at 18720

Blue

L'157
HPRT
Apa/Pvu

Take ✓ 12 µl

hrG Nct L'119 Blue (6)

✓ 82 µl

ddw

✓ 12 µl 10X NEN buffer 4

✓ 12 µl 10X Alb

✓ 2 µl (200) Apa I hot 30 (100/µl)

37° 2:20 pm to 3:20 pm

~~100/µl~~

Then plus 12 µl RI salts

3:27 pm to 4:30 pm

To cut a near 5.9 kb Apa frag⁵

Pvu I

✓ 4 µl (200)

lot 28 (50/µl)

On 0.8% Hx4 1 v/can

① Dye
AG-MM
110 slots L'157 HPRT Apa/Pvu
AG-MM
Dye

2nd Blue 40%

Recover into 60 µl 1X R/salts

L'157
HPRT
Apa/Pvu Frag

Then + 2 µl Pvu I (100) lot 28 (50/µl)

37° 10:54 am to 1:30 pm inactivated 80°C 20 min - 85°C

Ligation

L'157
L'16^N

Take ✓ 2 µl L'157 BS Apa CIAA

✓ + 4 µl L'157 HPRT Apa/Pvu Frag

✓ + 3 µl ddw

✓ + 1 µl 10X P95 lig² buffer RT 4:05 to 5:05

+ 1/2 µl K'99 T4 ligam

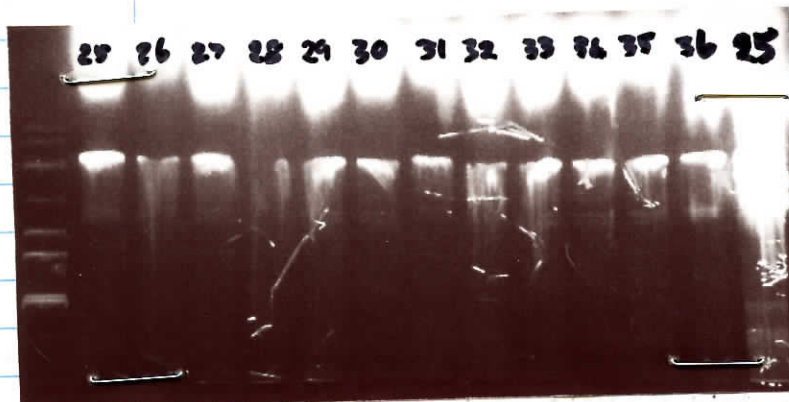
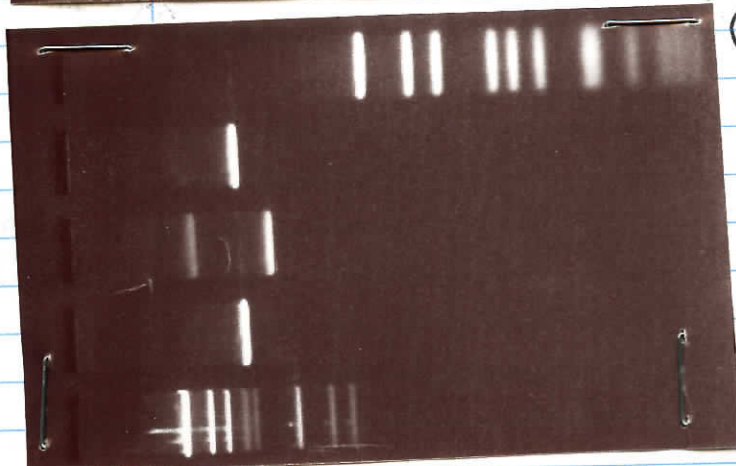
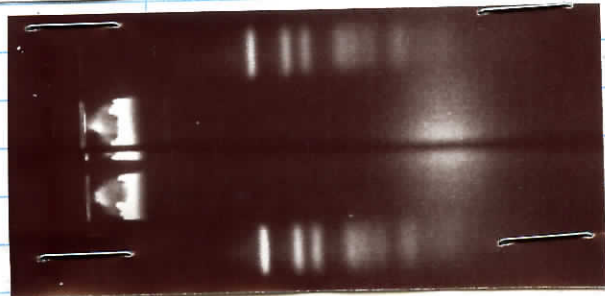
37° 5:05 to 7:05

power went off

152

L158 Blue Aff Eco Aff	
29702	TTA AGG GGA ATT CCG
12	
58%	
L158 Red Aff Eco Aff	
29703	TTA ACC GAA TTC CCC
12	
58%	

Redesigned
to ↑ GC content
but prevent
hair pin loop.



See
① * below

②

② Confirms ~ no EcoRI site
Go to new linker!

③ V. dirty gel
Still none. Go
back to ligation
L143 & screen 48 more.

3:30pm
Dye
W
PCR
L159 Eco Aff
L159 Eco
L159 Aff
AG
W
Dye
W
AG
(25)
EcoRI
on L153 mini
(36)
(25) Vnat
Dye

3/10
RNA
off

The
0.8/4
1.6
B

*
Per
all
str
in

Take 3 μl L151 YN_{seq} Aff II (isolated from t)

L158
L160
YN Eco

Compare
L161
Rigat

4 μl L158 Blue Aff Eco oligo
4 μl L158 Red Aff Eco oligo
6 μl ddw
2 μl 10x p95 lig² buffer
1 μl T4 ligase K99

Yam

Fr
15
9:55am

Minus (1) - (12) - none cut with Eco RI

Wed. July 25th3/10
RNAseOn 6:30 pm YN pEA frag^b

①

Dye

AGR

0.8% Hx4

0.5 v/cm

2/12
26/5T.H. L'153 Age/Eco
digest of #13.

Yellow 60%

ACR

Dye

Thurs July 26th3 frag^b

higaba

0.8% Hx4

Max

Take

pe L'149 BMHC PRO Age/Bam

pe L'149 BS Eco/Bam

pe L'159 YN pEA Age/Eco

pe d+w

pe p95 10x hig. buffer

pe T4 ligase K'99

L'159

L'159

BMHC YN

* Very strange, so little cutting with Age + Eco. Re-lett with
 individual enzymes & joint w/age L'153 pool.
 Perhaps it is all a single strand material in Clontech.
 Take 1/2 pe L'153 pool YN Eco
 ✓ 24 pe 10x RI salt
 ✓ 24 pe 10x Alb
 ✓ 10 pe d+w ✓ 180 pe d+w
 ✓ +1.2 pe Eco RI hot 29 (240) (200/pe)
 ✓ 12 pe (240) Age I hot 34 (200/pe)

L'159
Eco

16 pe

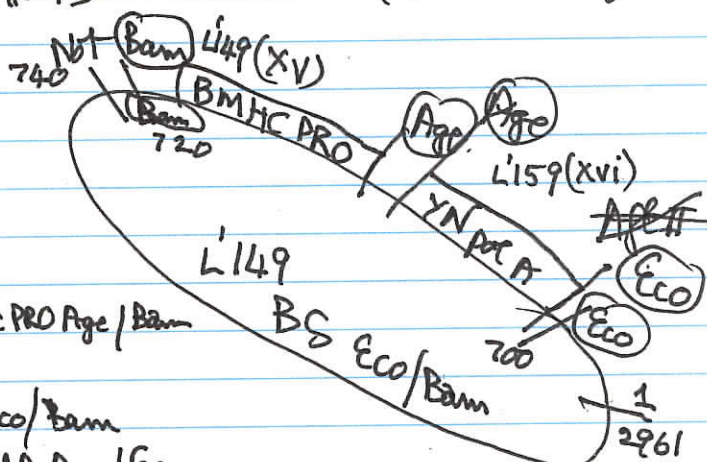
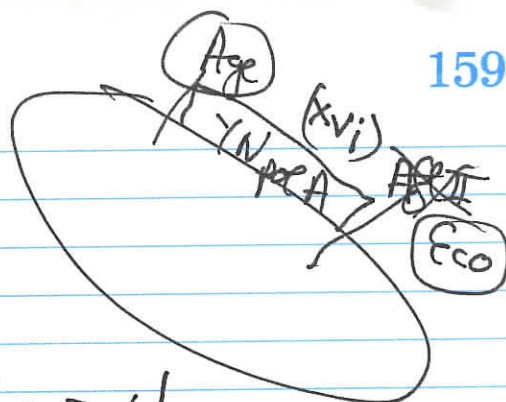
L'159
Age

16 pe

L'159
Eco/Age

37° 10:18 am

- 23 pm



Meanwhile
 re-design
 oligos. Perhaps
 too low Tm.