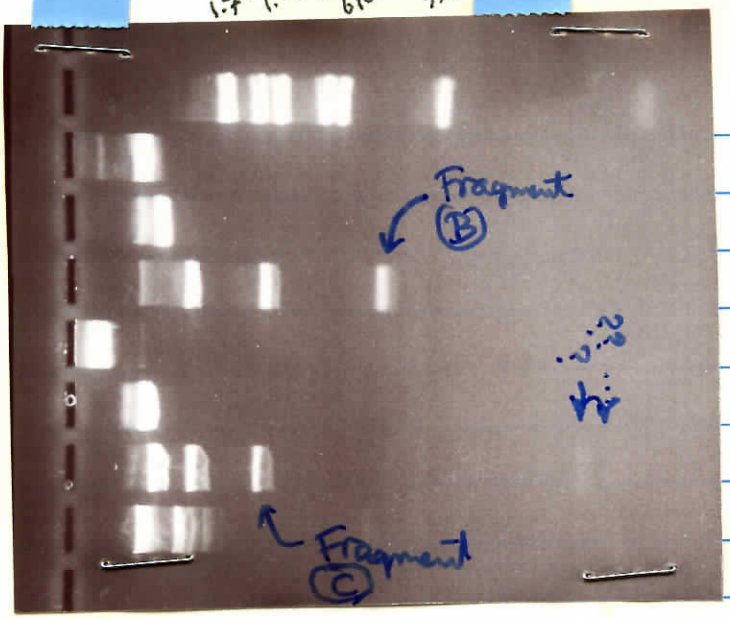
Smithies - May 1998

OLIVER SMITHIES
DEPT. OF PATHOLOGY
CB# 7525 UNC at CHAPEL HILL
NC 27599-7525

B C

(1)



Page

Conclusion: Cla I cuts both plasmids completely & singly. Fragment (B) is secure with 2 more Xho cuts. Fragment (C) has a potable problem — more Hind cuts than expected — so cannot exclude a Hind site close to Cla.

Sequence of PMP8 SKB shows that it is OK

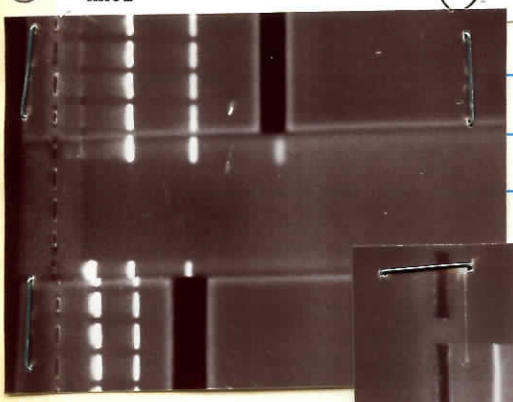
Hind (C) Cla
3055
3017
3203
Hind III
Eco RV 4472, Eco RI end 4495 4524 ← Ex Cla

(Circular) MAPPLOT of: PMP8SKBgcg.seq. ck: 1330, 1 to: 12720 May 29, 1998 16

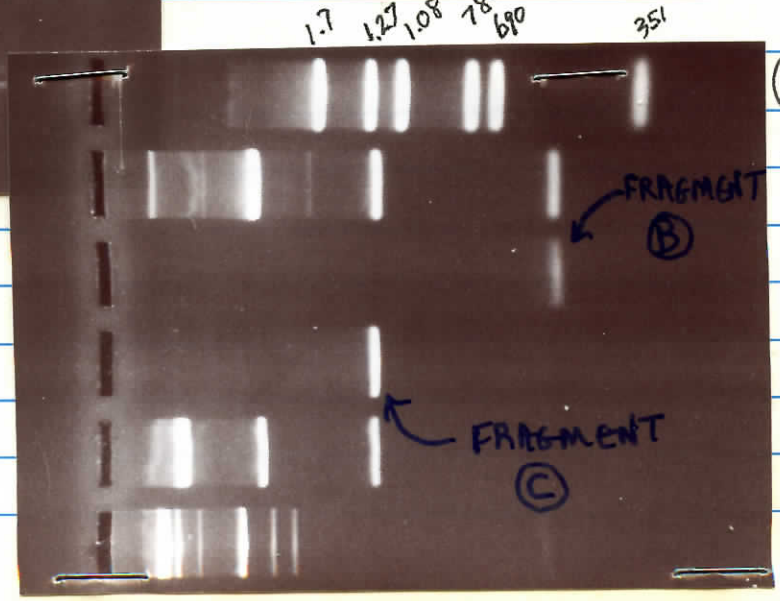
2000 4000 6000 8000 10000
2397

ClaI
EcoRI
EcoRV
HindIII
XhoI

(2)



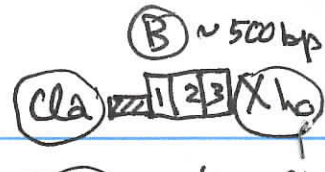
(2) Good cuts



(3) Good recovery of (C); adequate of (B).

Thurs. May 28th

Fragments B & C



Take 20 µl 10×NEB buffer 4 (KCl) | 20 µl 10×NEB buffer 4
153 µl dd w + 20 µl 10×BSA | 154 µl dd w + 20 µl 10×BSA
7.1 µl G'149 pMNI (5 µg) | 6.1 µl G'159 pMP4 (5 µg)
5 µl (25u) Cla I Lot 33 (50/µl) | 5 µl (25u) Cla I

37° 5:10 pm to 7:10 pm

~0.25 µg/10 µl

10 to 8

H'1 Pre B

H'1 B Post Cla

to freezer

H'1 Pre C

H'1 C Cla.

H'1 C Post Cla

Fri. May 29th

On 1:47 pm 3:50 pm 1.5 to 3V/cm Cla digests ppt.'d back into 150 µl 1×RI salts

Dye
W
ACR MM
B Post Cla/Xho
H'1 B Conc
H'1 C Conc
C Post Cla/Hind
ACR MM
W
Dye

+1 µl (20u) Xho I Lot 35 → H'1 B Cla/Xho

+1 µl (20u) Hind III Cla/Hind Lot 50 → H'1 C

10 to 8

H'1 B Post C/X

H'1 C Post C/H

37° 10:31 to 1:47 pm

2

Dye
Water
9 slots
B
Water
Dye
Water
9 slots
C
Dye

H'1 B Conc → into 60 µl 0.001M EDTA

H'1 C Conc → into 60 µl 0.001M EDTA

On 4.40 pm ~0.5 V/cm.

B C

2 (1)

Conclusion Sal worked fine in SSV 9. None of mums are cut - also mom bare nicked. Try a different approach: →

(1)

① 8:05 pm
on 11.0am 0.8% H×4 2V/cm → higher
0.5

Xba Hind Xba Sal Bam

Saturday May 30th

ΔSSV9 with H, S, B linker (cntd.)

Dye
W
1 — Processed G'162 minis to 0.001M EDTA.
2 — Screen first with Sal I – ΔSSV9 without linker will have no Hind, Sal or Bam – SSV9 has 1 of each.

Sal I digest — Take ✓ 52 μl 10×R1 salts
✓ + 463 μl ddw
✓ + 10 μl (100 u) Sal I Lot J99 (10 u/μl)
23
24
pSSV9/Sal ✓ – 20 μl aliquots – each + 1 μl G'162 minis 1–24
AGMM → 1 with ~ 1/3 μl pSSV9 G'161
W
Dye — 37° 6:35pm to 7:35pm

Sun. May 31st

The present ΔSSV9 approach has difficulties in later proving that both ITR's are intact (double Xba cut).
∴ change tactic → change one Xba site to Xba Hind & the other to Xba Bam → keep SSV9 intact
Most economical way is probably single cut Xba → make sets of Xba Bam & Xba Hind; sort into opposite site changes → combine as Cla I/Sal I fragments. Or do two consecutive changes, one single site & other complete.
Linkers for Xba → Bam & Xba → Hind

19125
Oligo #
Date made:
LENGTH: 14
% G+C: 57%
5' Oligo Name: H'3 Xba → Hind 3'

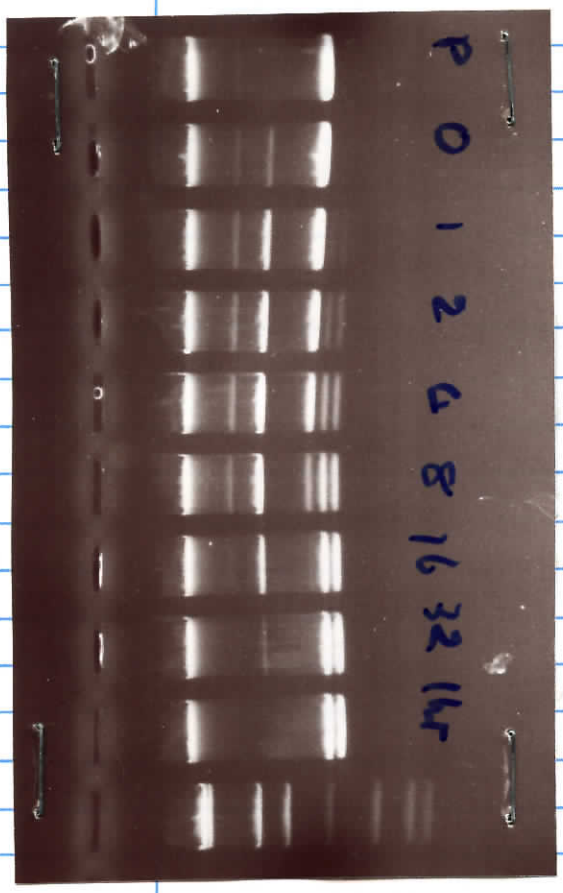
CTA	GGC	AAG	CTT	GC

19126
Oligo #
Date made:
LENGTH: 14
% G+C: 57%
5' Oligo Name: H'3 Xba → Bam 3'

CTA	GGA	GGA	TCC	TC

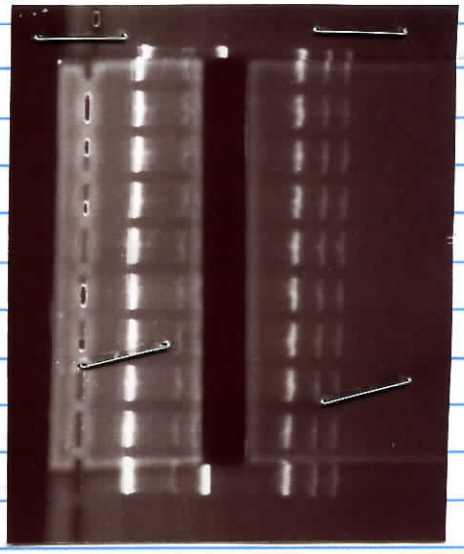
Thrown out ele H'20

(1)



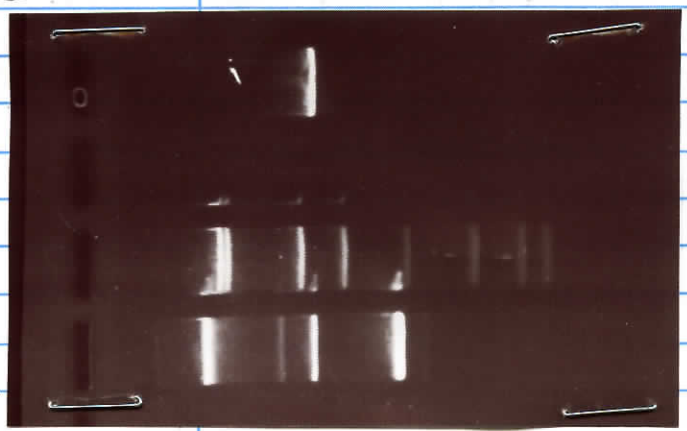
(1) Conclusion Good range. If repeat is req'd pick 4 min.

(2)



(2) Conclusion – adequate cut

(3)

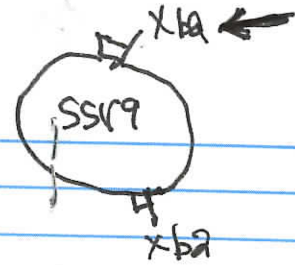


(3) Conclusion: Proceed – a good single cut product

(1) On 4:10 pm
Off
0.8% 11x14 Max

Dye
Pre
0
1
2
4
8
16
32
1hr
AGMM
Dye

Sunday May 31st



Single cut Xba SSV9

13.2
Take { ___ μl (10 μg) G'161 pSSV-9
+60 μl 10x RI salts
+527 μl ddw
+0.5 μl Xba I lot 30 (20u/μl)
(10u)

H'5
SSV9/Xba

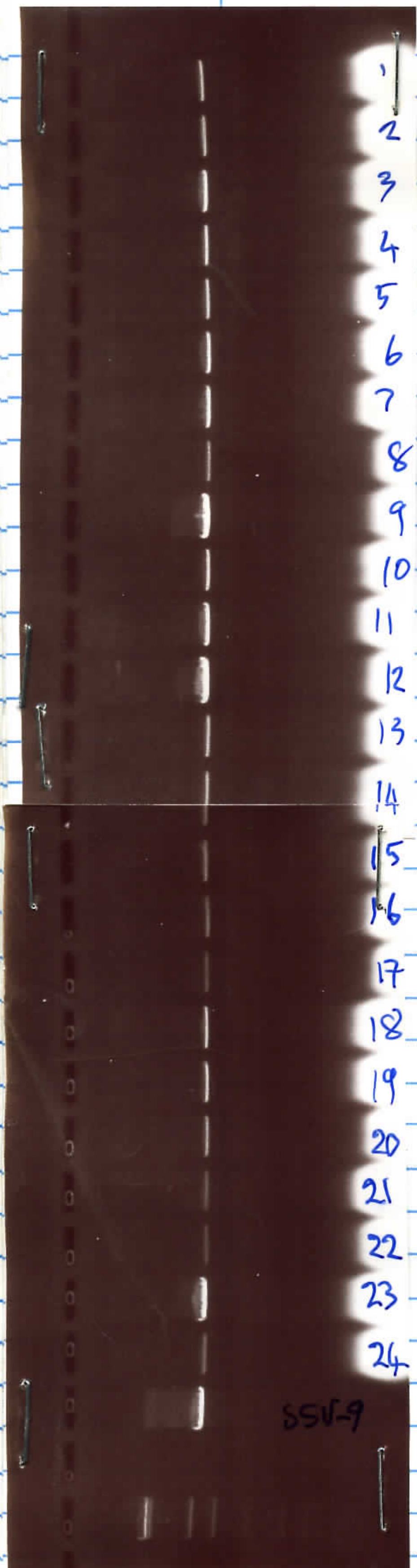
18 μl
Pre "0" 1 2 4 8 16 32 60
60 μl
2X sample
To 6 μl 10X PBS → to 80° for 3 min.

(2) On 6pm
Off ~9.0am
O/N 1.0
4x11 of Ag
0.8% Scap
Dye
W
} 10 slots – pool of 2, 4 & 8, topped off with 1
W
Dye

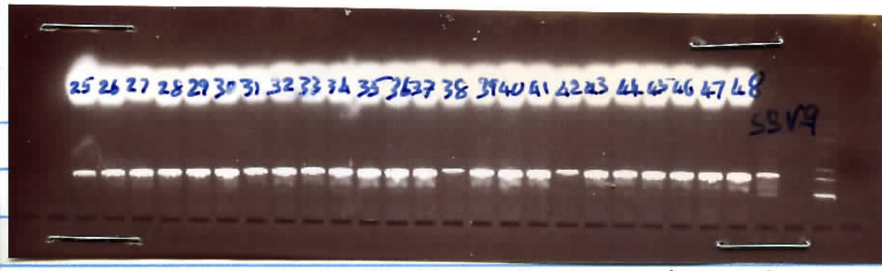
Prepared in 50 μl as single cut Xba
Vector conc

H'5 conc
Sc XVec

(3) 11:35 am
On 1 pm
Off
0.8% 4x14
O/N
Dye
W
H'5 conc scX Vec
W
AGMM
Time series 1 minute
W
Dye



②



① & ② Veryodd — none! ~~C~~ ditto ③ → ③

③

See H'18
for what is
available

Dye
W
49
50
|
61
62
SSV9
W
AgMM
W
Dye

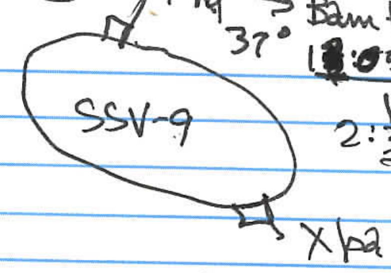
① On 2:30pm
Off 3:30pm
0.8% H×4 Max ~1 hour

37° Fri Plated 6pm – 8:30am
Processed ㊾–62 ~~Xba~~ → Bam H1
37° 1:05pm Sat
2:30pm

7

Tues. June 2nd

Site change in SSV-9



	②	
Dye	Dye	
W	W	
1	25	
2	26	
⋮	⋮	Take 12 μl (~1/3) H'5 conc single cut Xba Vector
23	47	✓ +1 μl H'3 Xba→Bam oligo
24	48	Heat to 60° for ~2 min.
pSSV-9	pSSV-9	✓ +1.5 μl 10× p95
W	W	✓ +1.5 μl T4 ligase lot 67
AGMM	AgMM	
Dye	Dye	15° 10am to 12:45pm; RT 12:45pm to 1:45pm; 37° 1:45pm to 2:40

(Left margin: ③ On 2:50pm Off 3:50 — Dye, W, 49, 50, …, 61, 62, SSV9, W, AgMM, W, Dye)

H'7 LIG^N box

to cold room

2 μl to 18 μl PBS, 80° 2 min., quick chill H'7 LIG^N/10

Second try with H'7 LIG^N Thurs. June 4

H'7^3 – 5pm Fri. (see H'7) – 10 μl + 1 μl 10×PBS 80° for 2 min, then to 80 μl DH5α

Transformation

4/10 H'7
DH5α 80 μl
Whole H'7 LIG^N/10 20 μl

H'7^2 5:15pm 0°C to 5:50pm

0° 2:48pm to 3:16pm — 4:47pm to 5:22pm
42° 90 sec.

H'7^2 Rest ⑨ 20, ③ 5, ① 20/16, 5/16 — 2:30pm
(~20) ⑥ ③ 5:30pm

H'7^2 picked all colonies
㊾ — ㊿1
62 — 72 from H'7 2:55pm

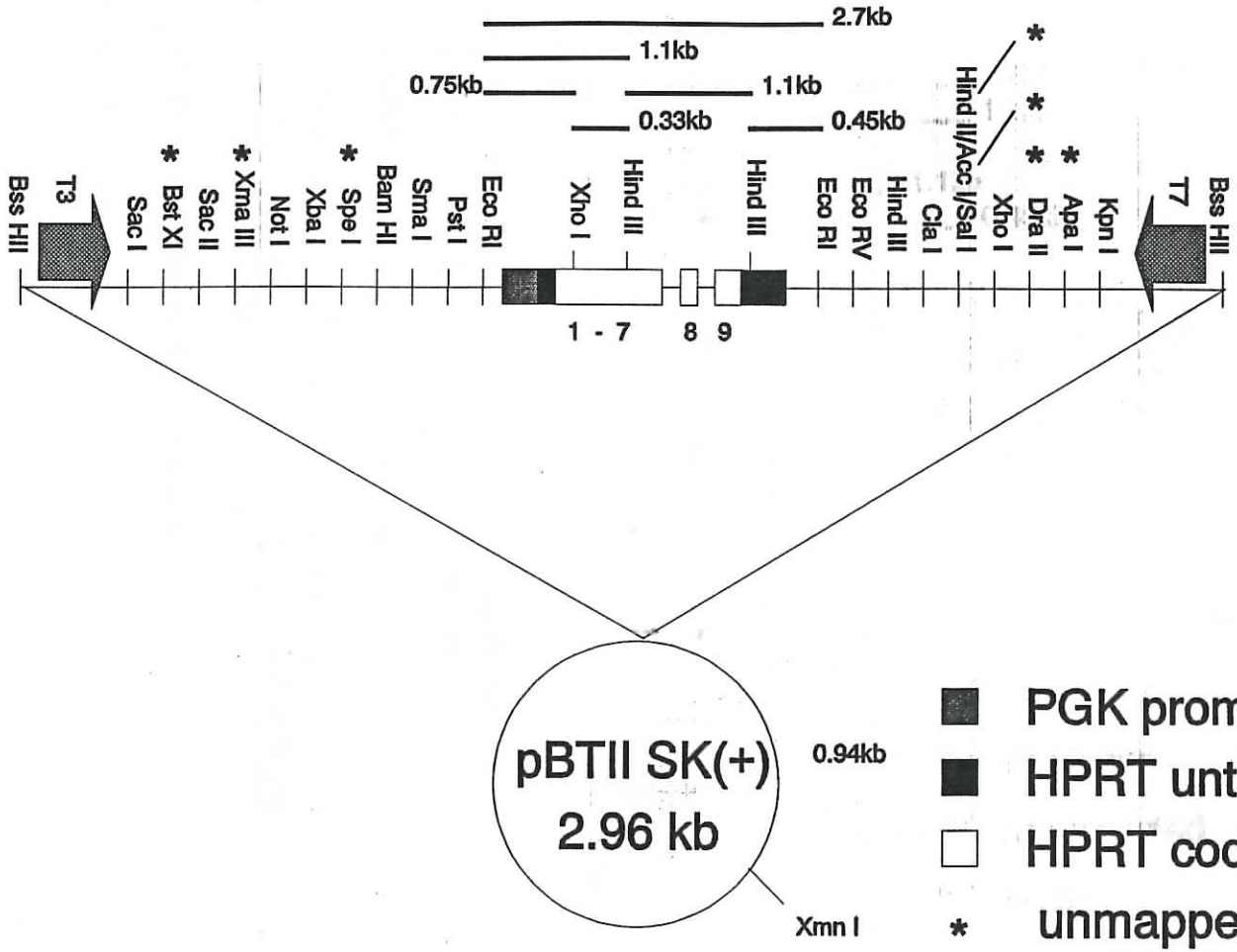
Plated 1× Amp NZY, Rest, 20, 5, 20/16, 5/16 37° 3:30pm

Minis H'7 ① — ㉔ 1× NZY Amp to 37° 10:15am 10am
㉕ — ㊽ 5.25pm Thurs Hi shake ~10pm cold

Processed to 50 μl.
✓32 μl ✓ Take 52 μl 10× R1 salts
✓285 μl ✓ +463 μl ddw
✓3 μl ✓ +5.3 μl Bam H1 Lot 77 (20u/μl) 4u/sample
20 μl aliquots → 1 μl minis → ½ μl G'161 pSSV9
37° 12:50pm to 2:10pm 37° ~12pm – 2pm

Expected lengths are: 8.3 kb unmodified
860bp + 7.5 kb 1 site
3.5kb + 4.9kb other site

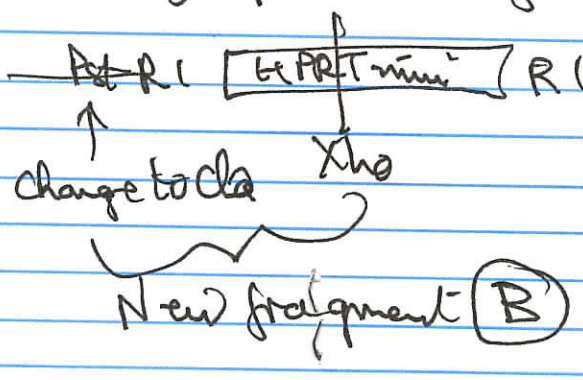
pBT/PGK-HPRT (RI)



Tues. June 2nd

Alternative HPRT minigene

Can use PGK promoter with 2 introns in place of pnNI, if nec.? The HPRT promoter in pBT/BWM 110 could be useful for h.r. construct. Gave to Seigo to grow up ⇒ consider. Will cut out new fragment B from pBT-HPRT(R1):-



Cell type switching

Cell A in steady state A with transcriptional/regulatory factor X in active form → with feedback to induce dephos.ⁿ of X-P.

Same cell in steady state B with factor X in inactive form (X-P) → with feedback to induce phosphorylation of X.

efferent
Renal arteriolar

SMC culture

Try to induce (by stretch or by low AT II) transition to (juxta)glomerular pericytic-like renin producing cells

afferent

Mon. June 1st

Some concrete thoughts on future work

Detection of atherogenic or protective mutations!

Cross F1 mutant (?) at test locus to heterozygotes to ApoE -/- & measure cholesterol (? on hi fat diet)

t^+/t^+ : $ApoE^+/ApoE^-$ Chol is 3X

t^+/t^{mut} : $ApoE^+/ApoE^-$ chol is 1.5X or 4.5X

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Minireview

The Enhanceosome and Transcriptional Synergy

Michael Carey
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University of California, Los Angeles
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Development of a complex eukaryote requires the differential transcription of over 50,000 genes in precise spatial and temporal patterns. One of the key problems in the gene expression field is understanding how an organism can achieve such diversity, while maintaining cell specificity and responding dynamically to its environment. One solution is to employ a limited repertoire of activators to minimize the complexity necessary to link related signaling pathways and to integrate diverse regulatory cues. The current view is that the cell accomplishes this by employing the principles of cooperativity and transcriptional synergy (Figure 1), where small combinations of ubiquitous, signal- and tissue-specific activators can be used to execute an exponentially larger number of regulatory decisions. Thus, an RNA polymerase II (pol II) enhancer responds to signals by organizing unique combinations of activators in a tightly clustered pattern that promotes their interaction and cooperative binding to DNA. The pol II transcriptional machinery, in turn, is designed to respond in a greater-than-additive or synergistic fashion only to multiple activators.

Previous studies from the Maniatis and Grosschedl laboratories on the IFNβ and TCRα gene enhancers, respectively, provided important biochemical details of how enhancer organization and cooperativity functioned to assemble activators into a nucleoprotein complex called the "enhanceosome." A key unanswered question was "how does the enhanceosome stimulate synergistic transcription and is the precise stereo-specific arrangement of activation domains necessary for the effect?" Recent biochemical studies reporting enhanceosome-activated transcription in vitro (Kim and Maniatis, 1997 [December issue of *Molecular Cell*]; Mayall et al., 1997) and the identification of activator "targets" within the transcriptional machinery (Bruhn et al., 1997; Merika et al., 1998 [January issue of *Molecular Cell*]) suggest that specific interaction surfaces are involved in synergy, and reveal new aspects of this regulation.

The Enhanceosome

The transition between the lysogenic and lytic states of bacteriophage λ in *E. coli* provided a paradigm for the role of cooperativity in gene regulation (Ptashne, 1992). Further studies established how cooperativity contributed to assembly of higher-order nucleoprotein structures, mediating what Echols termed "high-precision DNA transactions" during replication and site-specific

on the arrangement of activator recognition sites and the precise complement of bound activators, which together generate a network of protein–protein and protein–DNA interactions unique to a given enhancer. The free energy of enhanceosome formation is fine-tuned to the concentration of the relevant activators in a cell and their ability to engage in combinatorial interactions; subthreshold concentrations (see Figure 1), the absence of key activators, or altered positioning on the DNA prohibit cooperative binding. As illustrated in Figure 2, the enhanceosome displays two layers of "stereo-specificity" necessary for gene activation. In one, the contextual activator–activator interactions promote cooperative assembly of the enhanceosome on naked DNA or chromatin templates, an issue addressed by several previous studies from the Maniatis and Grosschedl labs (Giese et al., 1995; Thanos and Maniatis, 1995; Kim and Maniatis, 1997) and a recent study by Jones and colleagues (Mayall et al., 1997). In the other, the enhanceosome displays a specific activation surface that is chemically and spatially complementary to "target" surfaces on coactivators and the basal pol II transcriptional machinery,

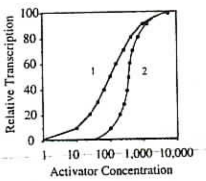

Figure 1. Manifestations of Synergy

There are two manifestations of synergy: The greater-than-additive transcriptional effect of multiple activator-binding sites on a promoter or enhancer, and the nonlinear or sigmoidal response of a gene to increasing activator concentrations. The vertical axis represents relative transcription. The horizontal axis denotes activator concentration (on a log scale). Curve 1 shows a standard parabolic response to increasing activator concentration for a reaction in which activators bind DNA noncooperatively and only a single activator is necessary to recruit the general machinery. Curve 2 shows the sigmoidal shape achieved by cooperative activator binding and by imposing a requirement for multiple activators, either the same molecule or combinations, to recruit the transcriptional machinery. The basis of the synergistic effect lies in a simple extrapolation of the Gibbs free energy equation, where the affinity of protein–protein interactions is exponentially related to the energy ($K = e^{-\Delta G/RT}$). The steepness of curve 2 would be influenced by the reciprocal cooperative effects of the transcriptional machinery and the activators. The curves are normalized to occupancy of 90%, or near maximal transcription. The enhanced sensitivity imparted by cooperativity is en-

Stryer "Biochemistry" 155

The Biochemical Basis of an All-or-None Cell Fate Switch in *Xenopus* Oocytes

James E. Ferrell Jr.* and Eric M. Machleder

Xenopus oocytes convert a continuously variable stimulus, the concentration of the maturation-inducing hormone progesterone, into an all-or-none biological response—oocyte maturation. Here evidence is presented that the all-or-none character of the response is generated by the mitogen-activated protein kinase (MAPK) cascade. Analysis of individual oocytes showed that the response of MAPK to progesterone or Mos was equivalent to that of a cooperative enzyme with a Hill coefficient of at least 35, more than 10 times the Hill coefficient for the binding of oxygen to hemoglobin. The response can be accounted for by the intrinsic ultrasensitivity of the oocyte's MAPK cascade and a positive feedback loop in which the cascade is embedded. These findings provide a biochemical rationale for the all-or-none character of this cell fate switch.

Fully grown *Xenopus laevis* oocytes are arrested in a state that resembles the G_2 phase of the cell division cycle with inactive cyclin-dependent kinase Cdc2 and an intact germinal vesicle. Exposure to the hormone progesterone induces oocytes to undergo maturation, during which they activate Cdc2, undergo germinal vesicle breakdown, complete the first meiotic division, and finally arrest in metaphase of meiosis 2 (*1*). Oocyte maturation is an example of a true cell fate switch; oocytes can reside in either the G_2 arrest or the metaphase arrest state for extended periods of time, but can be in intermediate states only transiently.

Progesterone-induced maturation is thought to be triggered by activation of a cascade of protein kinases—Mos, Mek-1, and p42 or Erk2 MAP kinase (MAPK). Progesterone causes the accumulation of Mos, which phosphorylates and activates Mek-1. Active Mek-1 in turn phosphorylates and activates p42 MAPK, which brings about activation of the Cdc2–cyclin B complex. Interfering with the accumulation of Mos (*2*) or the activation of Mek-1 (*3*) or p42 MAPK (*4*) inhibits progesterone-induced activation of Cdc2 and maturation, and microinjection of nondegradable Mos (*5*), constitutively active Mek-1 (*4, 6*), or thiophosphorylated p42 MAPK (*7*) brings about Cdc2 activation and maturation in the absence of progesterone. At some point in this chain of events, a continuously variable stimulus—the progesterone concentration—is converted into an all-or-none biological response.

Studies of the steady-state responses of the MAPK cascade in *Xenopus* oocyte extracts indicate that the cascade might contribute to the all-or-none character of oocyte maturation. In extracts, the response of MAPK to recombinant malE-Mos (a maltose-binding protein Mos fusion protein) is highly ultrasensitive (*8*), meaning it resem-

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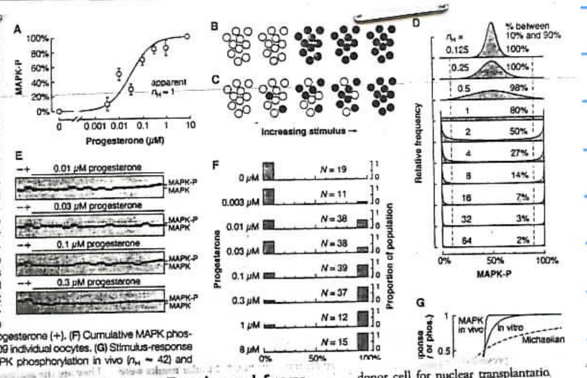

Fig. 1. Responses of oocytes to progesterone. (**A**) Overall responses. Each point represents a sample of 11 to 39 oocytes. Error bars denote two standard errors of the mean. MAPK-P, phosphorylated MAPK. (**B** and **C**) Two possible origins of a graded response. (**D**) Calculated distributions of oocytes incubated with a half-maximal stimulus for various assumed values of the Hill coefficient (n_H) for the individ-... ponses. The ... variability ... ere to corre-... = 1 curve ... immunoblots ... cytes treated ... ne. The first ... ch blot repre-... ated with no ...) or 8 μM progesterone (+). (**F**) Cumulative MAPK phos-... from N = 209 individual oocytes. (**G**) Stimulus-response ... for p42 MAPK phosphorylation in vivo ($n_H \approx 42$) and

BIOCHEMISTRY

The Era of Pathway Quantification

Daniel E. Koshland Jr.

On page 895 of this issue, Ferrell and Machleder (*1*) highlight a new era in our understanding of cellular metabolism. Knowledge of metabolic processes in cells can be roughly divided into three eras: the Era of Pathway Identification (1890–1950), the Era of Pathway Regulation (1950–1980), and the Era of Pathway Quantification (1980–?). In the first era, the individual steps in the biochemical pathways were identified. In now classical studies, the substrates, products, and enzymes of pathways such as glycolysis, fatty acid metabolism, and nucleic acid metabolism were identified by Emden, Meyerhof, Warburg, Kornberg, Cori, Brown, Goldstein, and many others (*2*). In the second era, the control of pathways through feedback, feed-forward, cooperativity, allostery, phosphorylation, and covalent modification was delineated by Pardee, Krebs, Fischer, Stadtman, Jacob, Monod, this author, and many others (*3*). In the third era, now in its childhood, the quantification of pathways is being examined to calculate the rates at which metabolites and substrates are produced and degraded in cells and in organs.

Ferrell and Machleder (*1*) examine the turning on and off of the cell cycle in oocytes, showing that this control process is quantitatively "ultrasensitive" (*4*) and that the enzymes responsible are the mitogen-activated protein kinase (MAP kinase) cascade. In their report, they examine this

An enhanced version of this commentary with links to additional resources is available for Science Online subscribers at www.sciencemag.org

process in intact oocytes (*1*); in a previous paper, Huang and Ferrell analyzed a cell-free system of the same MAP kinase cascade (*5*). Because individual enzymes of the cascade do not show cooperativity, it seems clear that some form of zero-order ultrasensitivity or multistep ultrasensitivity (*4*) is at work in this pathway, likely involving the kinetics of phosphorylation and dephosphorylation in the enzymes of the kinase cascade.

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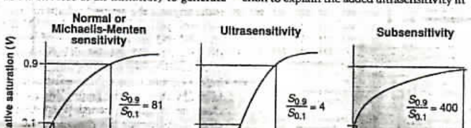
...pared to 2.8 for hemoglobin) to illustrate the powerful ultrasensitivity of this system. They also illustrate an important principle in this quantitation: Individual oocytes show an even greater ultrasensitivity than the ensemble as a whole, and

cal device or the melting solution, millions of molecules are involved, and therefore the sigmoid character of the response is obscured. As shown in the figure directly above, with smaller numbers of molecules (for example, four subunits in hemoglo-

Ultrasensitivity has been defined (*4*) as the response of a system that is more sensitive to changes in the concentration of the ligand than is the normal hyperbolic response given by the Michaelis-Menten equation. A Michaelis-Menten or hyperbolic response requires an 81-fold change in ligand S (the ligand can be a substrate or an effector of an inhibitor) to generate

they declare that this is caused by the individual systems having different midpoints to their saturation curves—that is, the average of highly sensitive systems with different $S_{0.5}$ values will give a much less sensitive overall response to a stimulus than would the individual systems themselves. They also postulate a feed-forward phenomenon to explain the added ultrasensitivity in

bin), the change is gradual but is markedly more sensitive than a hyperbolic curve to changes in the environment. Thus, a lack of knowledge of the mathematics would lead one to suggest that a drug acts only when it exceeds a threshold. In fact, low concentrations of a drug will have an effect, but it will be undetectable against the large background of a biological system (see figure below).



Some steps have already been made in the new Era of Quantification. Calcium spiking has been quantitated and allows the cell to set a sharp threshold to stimuli (*9*). The pathway of chemotaxis in *Escherichia coli* has been calculated in relation to the actual concentrations of the components of that response (*10*). The switch between the Krebs cycle and the glyoxalate shunt has been correlated quantitatively with the rates through each of the individual steps in the Krebs cycle and glyoxalate bypass (*8*). The use of energy in maintaining a phosphorylation system has also been quantitated (*11*). These are just a few illustrative examples of the quantitative conclusions that are possible when quantitation and the mathematics of quantitation become part of the arsenal of investigators of metabolic interactions. Investigators will be increasingly concerned with responses initiated by small fluctuations in the environment or cellular media, small changes in hormone supplies, and increases or decreases in enzyme levels, for example, as cells differentiate and dedifferentiate. We have gone from the era of "who" to that of "how" and are now entering the era of "how much." Ferrell and Machleder's report is a prime example of excellent data and thinking applied to a very important problem.

References

1. J. E. Ferrell Jr. and E. M. Machleder, *Science* 280, 895 (1998).
2. L. Stryer, *Biochemistry* (Freeman, New York, 1988) and historical references therein.
3. A. L. Lehninger, I. Nelson, M. M. Cox, *Principles of Biochemistry* (Worth, New York, 1993).
4. A. Goldbeter and D. E. Koshland Jr., *Proc. Natl. Acad. Sci. U.S.A.* 78, 6840 (1981); D. E. Koshland Jr., A. Goldbeter, J. B. Stock, *Science* 217, 220 (1982).
5. C. F. Huang and J. E. Ferrell, *Proc. Natl. Acad. Sci. U.S.A.* 93, 10078 (1996).
6. C. Bohr, K. A. Hasselbach, A. Krough, *Skand. Arch. Physiol.* 16, 402 (1904).
7. D. C. La Porta and D. E. Koshland Jr., *Nature* 3..., 286 (1983).
8. D. C. La Porte, K. Walsh, D. E. Koshland Jr., *J. Biol. Chem.* 259, 14068 (1984).
9. T. Meyer and L. Stryer, *Annu. Rev. Biophys. Biophys. Chem.* 20, 153 (1991); P. De Koninck and H. Schulman, *Science* 279, 227 (1998).
10. D. Bray, R. B. Bourret, M. I. Simon, *Mol. Biol. Cell* 4, 469 (1993).
11. A. Goldbeter and D. E. Koshland Jr., *J. Biol. Chem.* 262, 4460 (1987).

Cloned Transgenic Calves Produced from Nonquiescent Fetal Fibroblasts

...e B. Cibelli, Steve L. Stice, Paul J. Golueke, Jeff J. Kane, ...oseph Jerry, Cathy Blackwell, F. Abel Ponce de León, James M. Robl*

...ient system for genetic modification and large-scale cloning of cattle is of im-...e for agriculture, biotechnology, and human medicine. Here, actively dividing ...roblasts were genetically modified with a marker gene, a clonal line was selected, ...cells were fused to enucleated mature oocytes. Out of 28 embryos transferred ...cipient cows, three healthy, identical, transgenic calves were generated. Fur-...re, the life-span of near senescent fibroblasts could be extended by nuclear ...r, as indicated by population doublings in fibroblast lines derived from a 40-day-...al clone. With the ability to extend the life-span of these primary cultured cells, this ...would be useful for inducing complex genetic modifications in cattle.

...rch has been in progress for more ...a decade to develop a system for ...c modification and large-scale clon-... cattle (*1*), an important species in ...lture, biotechnology, and human ...ine. In the initial work on cloning, ...yonic blastomeres were used as donor ...because they were thought to be ...vely undifferentiated, readily repro-...ned, and likely to support full-term ...opment of the fetus (*2*). Initial ef-... at refining the methodology of nucle-...nsfer resulted in significant, but lim-... improvements in efficiency, and at ...only a few identical calves could be ...uced from a single donor embryo be-...e of the limited number of cells in the ...embryo (*3*). The next step toward ...nding the potential of cloning was ...development and use of embryonic ...cells as a source of donor nuclei. ...bryonic stem cells are derived from the ...ner cell mass of an early embryo and are ...ought to be relatively undifferentiated.

In addition, mouse embryonic stem cells divide indefinitely in culture without differentiation and can be readily genetically modified (*4*). Embryonic stemlike cells have been developed in the bovine (*5*) and have been used as a source of donor nuclei in nuclear transfer, but they only supported development of fetuses to 60 days in vivo (*6*). To date, a source of cells that can be used for genetic modification and large-scale cloning in cattle has not been found.

Other research in nuclear transplantation has shown that the cell cycle stage of the donor cell affects the extent of development of the embryo after nuclear transfer. When the donor cell is fused to the recipient oocyte, which is arrested in the second metaphase in meiosis, the nuclear envelope breaks down and the chromosomes condense until the oocyte is activated (*7*). This condensation phase has been shown to cause chromosomal defects in donor cells that are undergoing DNA synthesis (*7*). Donor cells in the G_1 phase of the cell cycle (before DNA synthesis), however, condense normally and support a high rate of early development (*7*).

In previous work in the sheep, it was suggested that arrest in G_0 (by serum starvation) was the key in allowing donor somatic cells to support development of embryos to term (*8*).

Our rationale in selecting an optimal donor cell for nuclear transplantation was that the cell should not have ceased dividing (which is the case in G_0) but be actively dividing, as an indication of a relatively undifferentiated state and for compatibility with the rapid cell divisions that occur during early embryo development. The cells should also be in G_1, either by artificially arresting the cell cycle or by choosing a cell type that has an inherently long G_1 phase. We chose fibroblasts from fetuses because they can grow rapidly in culture and have an inherently long G_1 phase (*9*).

Fetal fibroblasts were isolated from a day 55 male fetus (Fig. 1A), cultured in vitro, and passaged twice before being transfected with a marker construct consisting of a β-galactosidase–neomycin resistance fusion gene driven by a cytomegalovirus (CMV) promoter (pCMV/β-GEO) (*10*). Cells were selected with neomycin for 2 weeks, and five neomycin-resistant colonies were isolated and analyzed for stable transfection by polymerase chain reaction (PCR) amplification of a segment of the transgene (*11*) and by assay of β-galactosidase activity. Colony CL1 was chosen for nuclear transfer experiments. These fibroblast cells

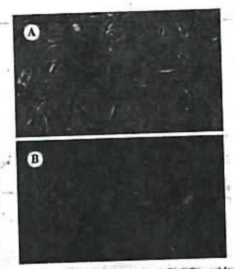


Fig. 1. Transgenic fetal fibroblast CL1-5 used for nuclear transplantation (**A**) phase contrast (×100). (**B**) Labeling of CL1-5 fibroblast cell line with PCNA monoclonal antibody (Sigma, St. Louis, MO) and FITC-conjugated secondary antibody (magnification × 200).

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N.M. - return key!

Wed. June 3rd

All or none cell type switching (H'10 cont'd)

Several recent papers suggest that the cytoplasm may be able to switch the nuclear behaviour in a nucleus transfer expt. By inference external factors may be able to cause switching between cell types, e.g. smooth muscle cell to fetroblast or to renin-producing cell.

The key references are to left.

Consider carrying out tests such as:-

Juxtaglomerular cells making renin (e.g. ♀)

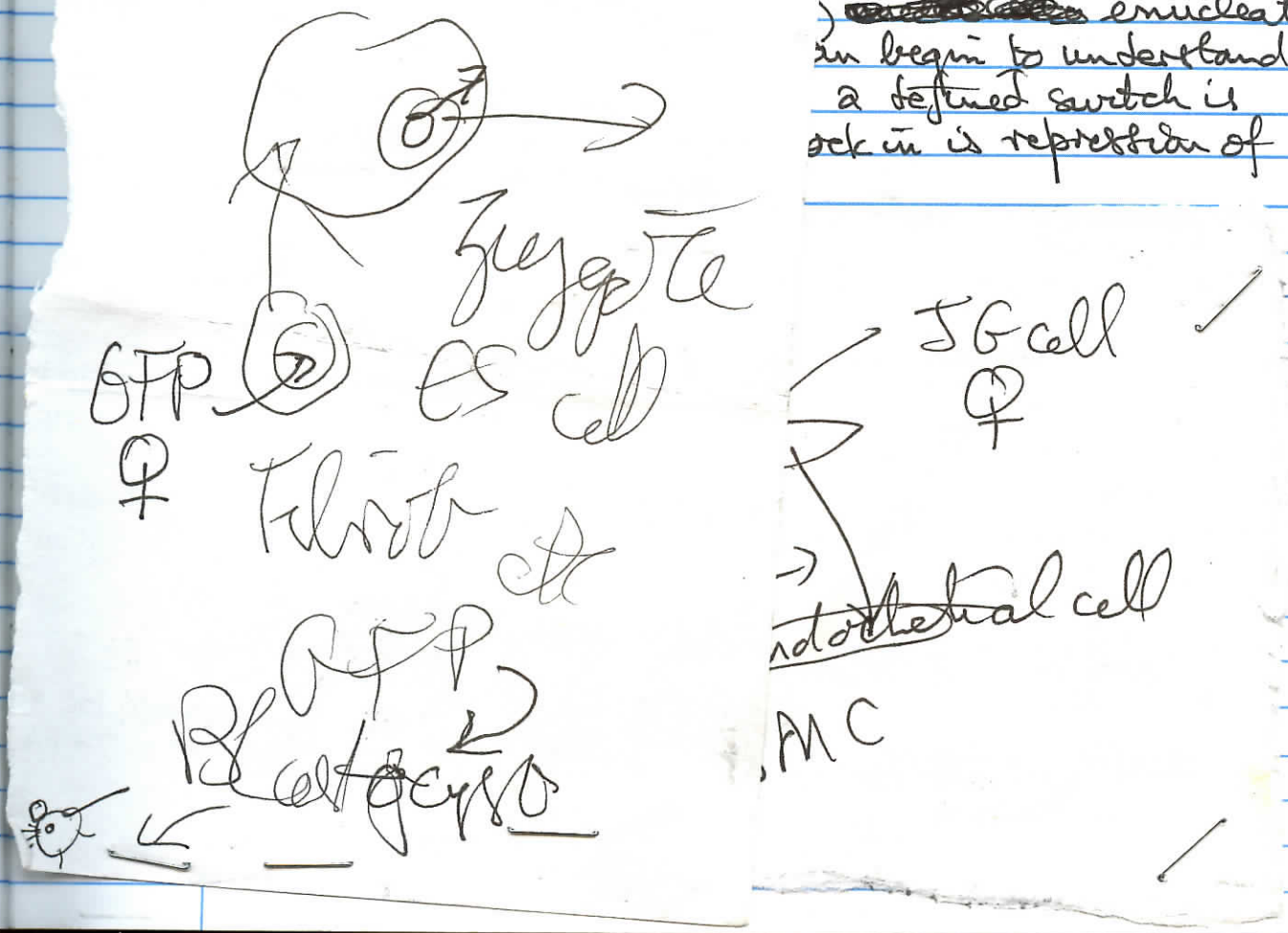
♂ ES cell nuclei (or whatever) to divide as J.G. cells

enucleated

in begin to understand

a defined switch is

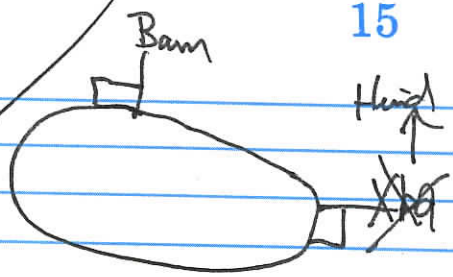
ock in is repression of the



Counting chickens!

Thurs. June 4th

Second Xba → Hind on pSSV9



Take 13 µl 10xR1 salts
116 µl ddw
12 µl XbaI (24u) lot 30 (20u/µl)

H'15 Xba

30µl 30µl 30µl 30µl

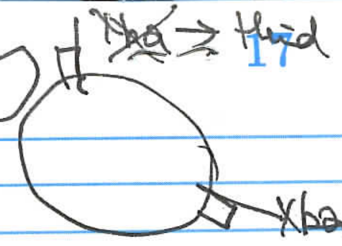
37° — to —

1.5 1µl H'7 mini gel run on the Xba digests, H'7 Don't need to inactivate XbaI

10µl Each + 1µl H'3 Xba → Hind oligo
+ 1.4 µl p95 10x ligase buffer
+ 1.5 µl T4 ligase lot 67

15° — to —

Fri. June 5th (See H'20)



Back-up 1st Xba → Hind

Take 12 μl (~1/3) H'5 conc single cut Xba Vector

✓ + 1 μl H'3 Xba → Hind oligo [H'17 LIG^n]
✓ + 1.6 μl 10× pp5
✓ + 1.6 μl T4 Ligase lot 67

15° 9:20am to 12:20pm ; RT 12:25pm to 2pm ; 37° 2pm to 3pm to cold

4 μl to 36 μl 1×PBS 75-80° 2 min, quick chill

whole Sat ~10 μl + 1 μl 10×PBS 70° 2 min.

to 11am

H'17²

Plated 2× Amp (supply) 1× NZY Transformation
37° 2:30pm Saturday
Rest 20 5 20/16, 5/16 (3)
Many (15)

4/10 H'7 DH5α — 80 μl ✓ — ✓ 80 μl
Diluted/Heated lig^n — 40 μl ✓ — 11 μl whole
0° 3:18pm to ~4.15pm ✓ — 1:22pm to 2:02pm

42° 90sec ✓

Only 3 colonies (2) (1)

(H'17) Plated 1× NZY Amp Rest, 20, 5, 20/16, 5/16
37° 4:30pm — Noon

(H'17²)

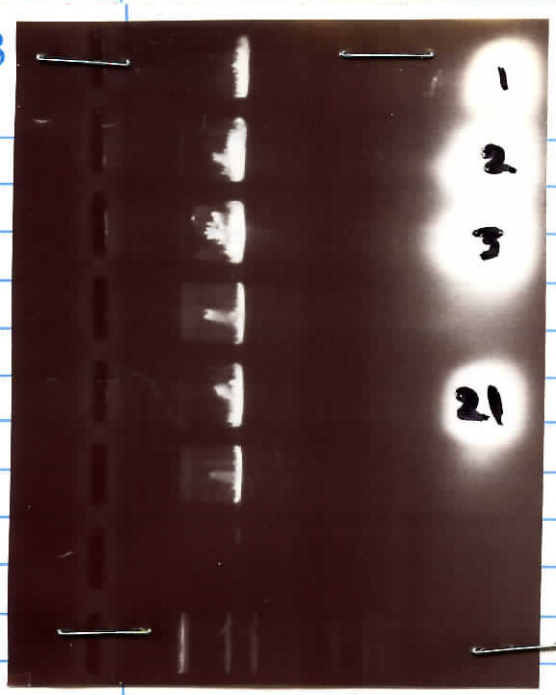
Fri June 5th Back-up 1st Xba → Bam

Long H'7 lig^n whole (10 μl) + 1 μl 10× PBS 70° for 2 min then rapid cool → to 80 μl 4/10 H'7 DH5α → transformed

Many (~20)

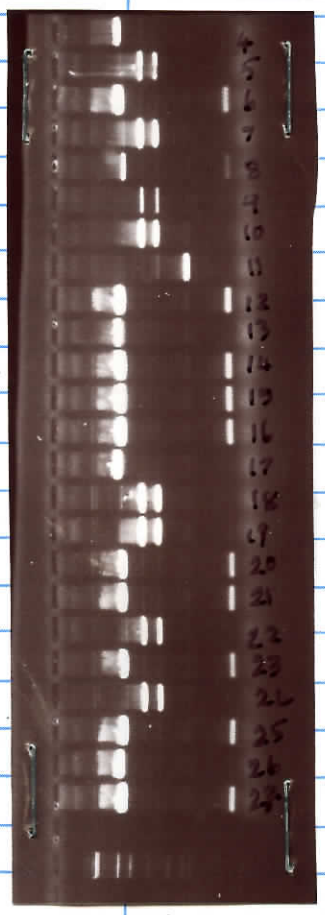
(H'7³) 1× NZY Amp Rest, 20, 5, 20/16, 5/16 5pm

Sat June 6 Minis H'7³ (1) — (21) Xba → Bam 37° 4:25pm to 11am
→ H'17 (1) — (3) Xba → Hind



① ditto!!! still negative

If the next 25 don't work, remake the oligos.
Possibly oligos are interchanged - Text Bam with Hind & vice versa.



② Conclusion Extents of H17² minis
Xba → Bam: ① & ② are Xba 4494 → Bam as are also ⑤, ⑦, ⑨, ⑩, ⑱, ⑲, ㉒ & ㉔
Xba 184 → Bam are represented by ⑥, ⑧, ⑫, ⑭, ⑮, ⑯, ⑳, ㉑, ㉓, ㉕ & ㉗

H17² Pool Minis
Xba → Bam
~~4494~~
5, 7, 9, 10, 18, 19, 22, 24

H17² Pool Minis
Xba 184 → Bam
6, 8, 12, 14, 15, 16, 20, 21, 23, 25, 27

H17² Xba 4494 ⑤⑨ Hind Saved

H17 Pool Minis Xba 4494 → Hind
49, 52, 54, 56, 57, 58

① 3:25pm / 4:20pm / 0.8 / 0.8 10 H'x4 MAX

Sun. June 7th

But see H2O

Xba → Hind

Dye / W / 1 2 3 H'17 Hind / pSSV9 / 21 H'7³ / pSSV9 Bam / W / Marker / W / Dye

pSSV-9 Xba(1) → Hind

Processed H'17 ① – ③ + H'7³ ㉑ to mini (Rest of H'7³ hold at KOAc).

H'7³ Take ✓13 μl 10x R1 salts
✓117 μl ddw
✓80 μl ... 45 ✓
✓+1 μl (20u) Hind III Lot 50 ... + ½ μl (10u) Bam H1 Lot 77
20 μl ... 20 ... 20

H'17 Mini 1 μl: ① 17, ② 17, ③ 17, ½ μl SSV-9 H9, Mini H'7³ 1 μl ㉑ 7, ½ μl SSV-9 G'161 B9

37° 12 noon – 3:15pm

② H'17² minis ④ — ㉗ 1x Amp NZY 4:30pm Sun – 9:30am

10:30am / 11:30am / 0.8 / 0.8 10 H'x4 2 μl RNAse/ml MAX

Processed to 50 μl

Monday June 8th

Xba → Bam (contd.) ↑ Revised expectation

Dye / W / 4 5 ⋮ 26 27 H'17² minis Bam HI / W / Marker / W / Dye

✓Take 52 μl 10x RI salts
✓+ 463 μl ddw
+ 5.3 μl Bam HI Lot 77 (20u/μl) 4u/sample
20 μl aliquots + 1 μl H'17² minis ④ – ㉗

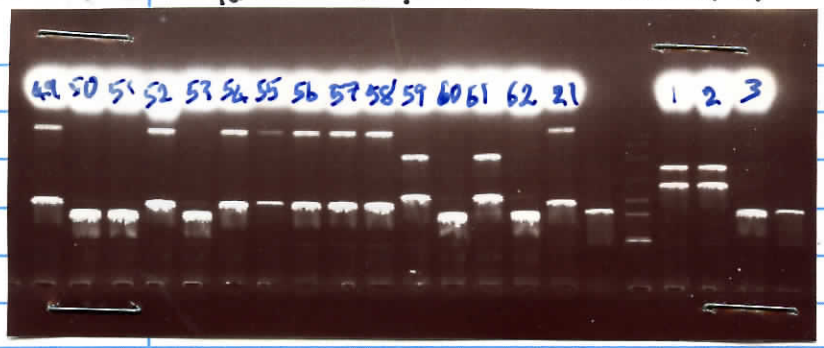
37° 6:35pm/6:40 9:15pm

Tues. June 9th

Catch-up on H'17² Xba → Bam ← Revised expectation

19/24 are converted!

Hind H'17² Bam H'17



(1) Conclusion: Tubes were mixed at some step. Both versions of Hind are available, only one of Bam. Proceed to isolate second Bam (from H'17²). Meanwhile from H'17² mini's can go on but remake oligos before Xba → Bam

Oligo # 190 (191)
Date made:
LENGTH: 14
% G+C: 57%
5' Oligo Name: H'20 Xba → Hind 3'
C T A | G G C | A A G | C T T | G C
Blue

Oligo # 91 (191)
Date made:
LENGTH: 14
% G+C: 57%
5' Oligo Name: H'20 Xba → Bam (Red) 3'
C T A | G G A | G G A | T C T | T C

H'17² (59) Saved as Xba 4494 → Hind See H'18

H'17² Pooled minis (49, 52, 54, 56, 57, 58) Saved as Xba 184 → Hind See H'18

(1) 1:35 pm 2:45 pm 0.8% HX4

Ma Tune 8th

Cross-cross tests

all were tested with Bam (H'6) (3)

H'17 (1) — (3) — putative Hind — all tested with Hind (H'18) (1) and were negative.

H'7² (49) — (62) — putative Bam — H'7³ (21)

∴ Set up reciprocal :-

Take ✓ 40μl 10×R1
✓ 360 μl ddw
✓ 320μl
✓ +4μl Hind III Lot 50 (20u/μl)

✓ 80μl + ✓ 1μl Bam H I Lot 27 (20u/μl)
20μl

H'17 (1) (2) (3) pSSV9

H'7² (49) - - - - (62) 21 pSSV9
H'7³

37° 10:40 am to 1:15 pm

Many are now positive

Result

Hind

H'7 — Xba

49 52 54
55 56 57
58 21

All H'7 are Hind III candidates

Xba 184 → Hind

Xba

H'7 — Hind

59 61

Xba 4494 → Hind

Hind Bam

Xba 184 → Bam

Xba

H'17 — Bam

1 2 5 7 9 10 18 19 22 24

Xba 4494 → Bam

Saved - see H'18

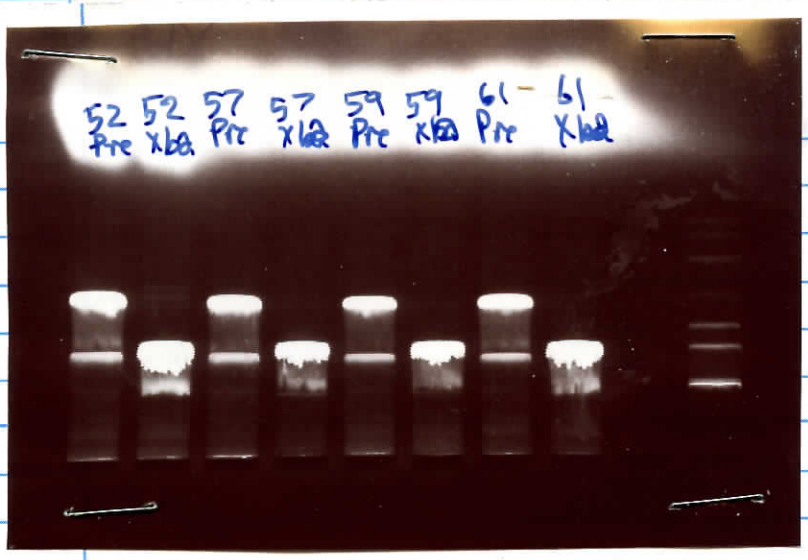
6 8 12 14 15 16 20 21 23 25 27

All H'17 are Bam H I candidates

Saved - see H'18

22

D



① Conclusion

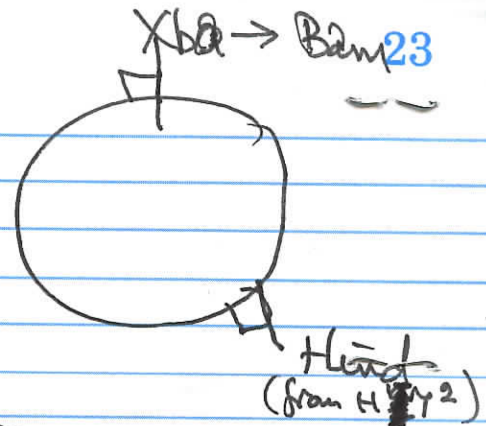
Cuts as expected. Products are good enough to proceed without gel purification.

① on 9:30am off 10:25 0.8 H'γ4

Dye
W
52 Pre
52 Xba
57 Pre
57 Xba
59 Pre
59 Xba
61 Pre
61 Xba
W
Marker
W
Dye

Mon. June 8th

pSSV-9 Second Xba to Bam HI



Take ✓ {48 μl 10×RI salts / 432 μl ddw}

✓✓ 20 μl **Pre** → 52, 57, 59, 61

H'γ2 minis 1 μl

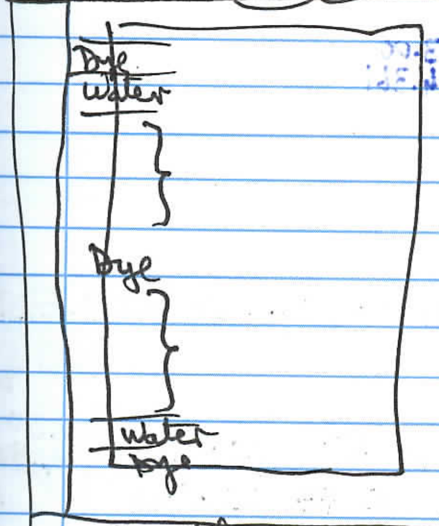
✓ 400 μl + 5 μl Xba I
lot 30
20u/μl
1μl
4/sample

Xba → ✓100 μl 52, ✓100 μl 57, ✓100 μl 59, ✓100 μl 61

H'γ2

37° 6:15 pm

9:15 pm ✓✓✓ 5 μl H'γ2 minis



Standby

Linker Ligation Tues. June 9th

Take ✓ 2 μl H'23/52 Xba ✓ 2 μl H'23/61 Xba
✓ + 2 μl H'20 Xba→Bam ✓ 2 μl oligo Red
✓ + 14 μl ddw ✓ 14 μl ddw
✓ + 2 μl 10×p95 buffer ✓ 2 μl
+ 2 μl T4 ligase 2 μl
lot 67

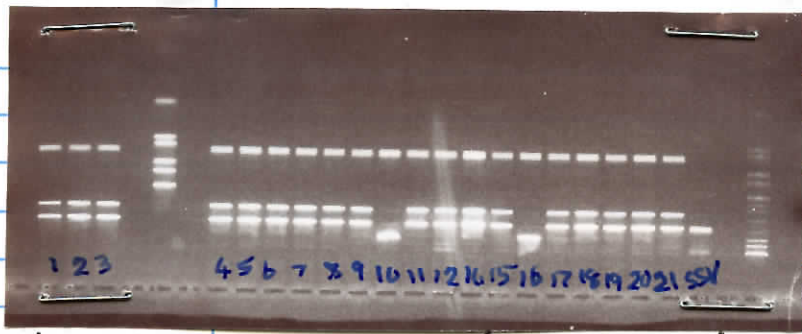
H'23 (52) LIG^N **H'23 (61) LIG^N**

15° 11:20am to 2pm

RT 2 to 3:40

37° 3:40 to 5:40pm — to cold

✓ 10 μl + 1 μl 10×PBS → Heated 70° → cooled



61

52

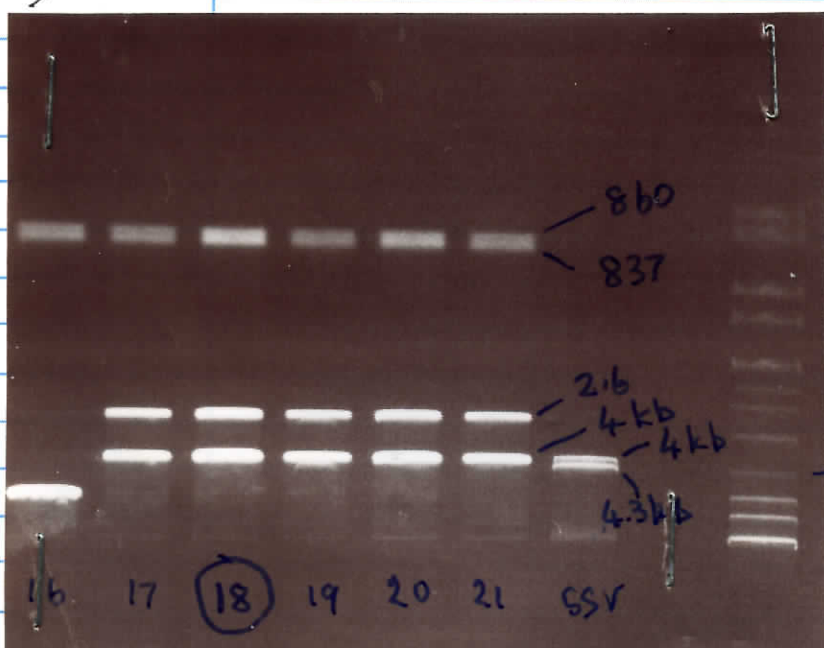
(1)

Conclusion: 18/20 are correct, & both varieties.

H'25 Pool Mini
Hind 4.484
Bam 184
1,2&3

H'25 (18)
Hind 184 Bam 4494

H'25
Pool mini
Hind 184 Bam 4494
4,5,6,7,9



– 3.00
– 4.361

Minis H'25 (1)(2)(3) and (4) – (21) processed to 50 μl 0.001M EDTA.

✓ 22 Take ✓ 22 μl 10×RI salts

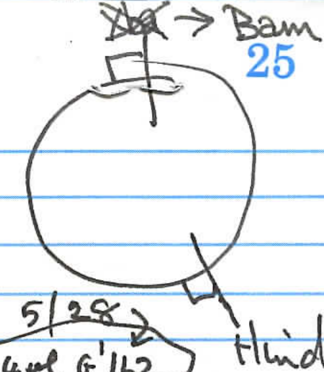
(10) ✓ 188 ✓ 188 μl ddw

(1) OK ✓ 2.5 ✓ 5 μl (100 u) Hind III lot 50 (20u/μl) | 100 4000 u/sample

✓ 2.5 ✓ 5 μl (100 u) Bam HI lot 77 (20u/μl)

20 μl aliquots + 1 μl each of H'25 minis. 37° 11:50am to 1.10pm

Error of (11)



① 1:40pm
2:45pm
1.5% H' ×1 1µg/ml
+2µl RNAse ~10V/cm

Tues June 8th

Second Xba → Bam (cut!)

Dye
W
① ② ③ ④ ⑤ ⑥1 Bam/Hind w/ ACR MM W
No 13 ⑤2 Bam/Hind ⑳ ㉑

Transformations

4/10 DH5α to

	(52)	(61)
	✓ 80µl	µl 46 ✓ +3.4µl G'162 5/28
	11µl ✓	11µl ✓ heated } +PBS H'23 ligation
0°C	5:50pm to	6:33pm
	42° ③ 90 sec ✓	
Plated	Rest (18) 20, 5, 20/16, 5/16 (52)	
pSSV-9X/stock 1x AMP N2Y	Rest 20, 5, 20/16, 5/16 (61) 37° 6:55pm	
Wet plates. ② ①		
left open for	later closure by J.H.	
	OK for (52); but (61) left overnight.	
	Tried H_2O/CO_2 incubator at 9.10am.	

W
Ag MM
W
Dye

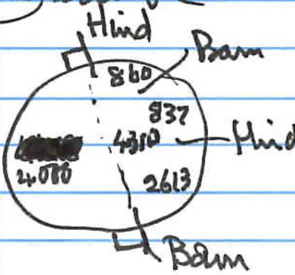
Minis ① ② ③ ④ — ㉑
from 61 — from 52 to 37°
37° at — RF at — 37° at
~12:15pm — ~12:30pm — 5:20pm
2:15 → rt

Transformation

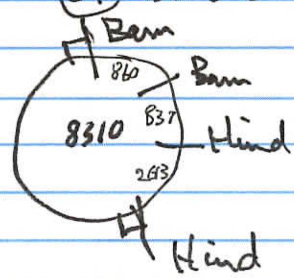
H'25 5/28 DH5α	80µl
→ to Overnite H'23 LIG^N	10+1 heated 10×PBS
0° 12:46pm to	Rest ~1:40pm
42°	90sec
Plated 2× Amp	N2Y
Rest 20, 5, 20/16, 5/16	37° 1:55pm
(20)	④

With Hind III + Bam HI expect
↓
40kb + 2.6kb + 860bp + 837bp
Control is pSSV9 + Xba i.e. G'161 pSSV-9 Xba stock

(52) will be

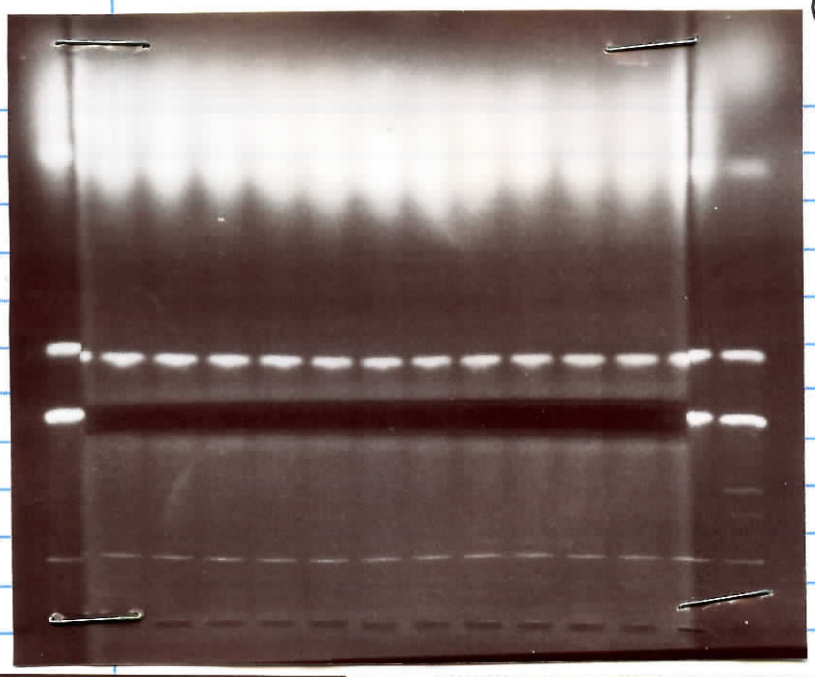


(61) will be

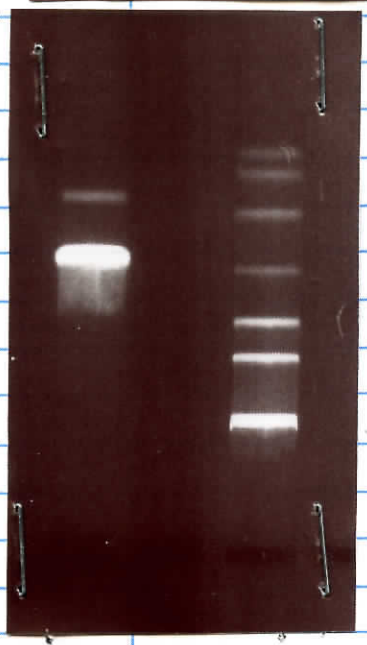


Can check with Bam & Hind alone later.

B C



① Conclusion: Good digest; good cuts



② Conclusion: Rather disappointing purity. The 2.6 kb insp. is H/B + will complete. Proceed anyways, but keep SSV H/B low

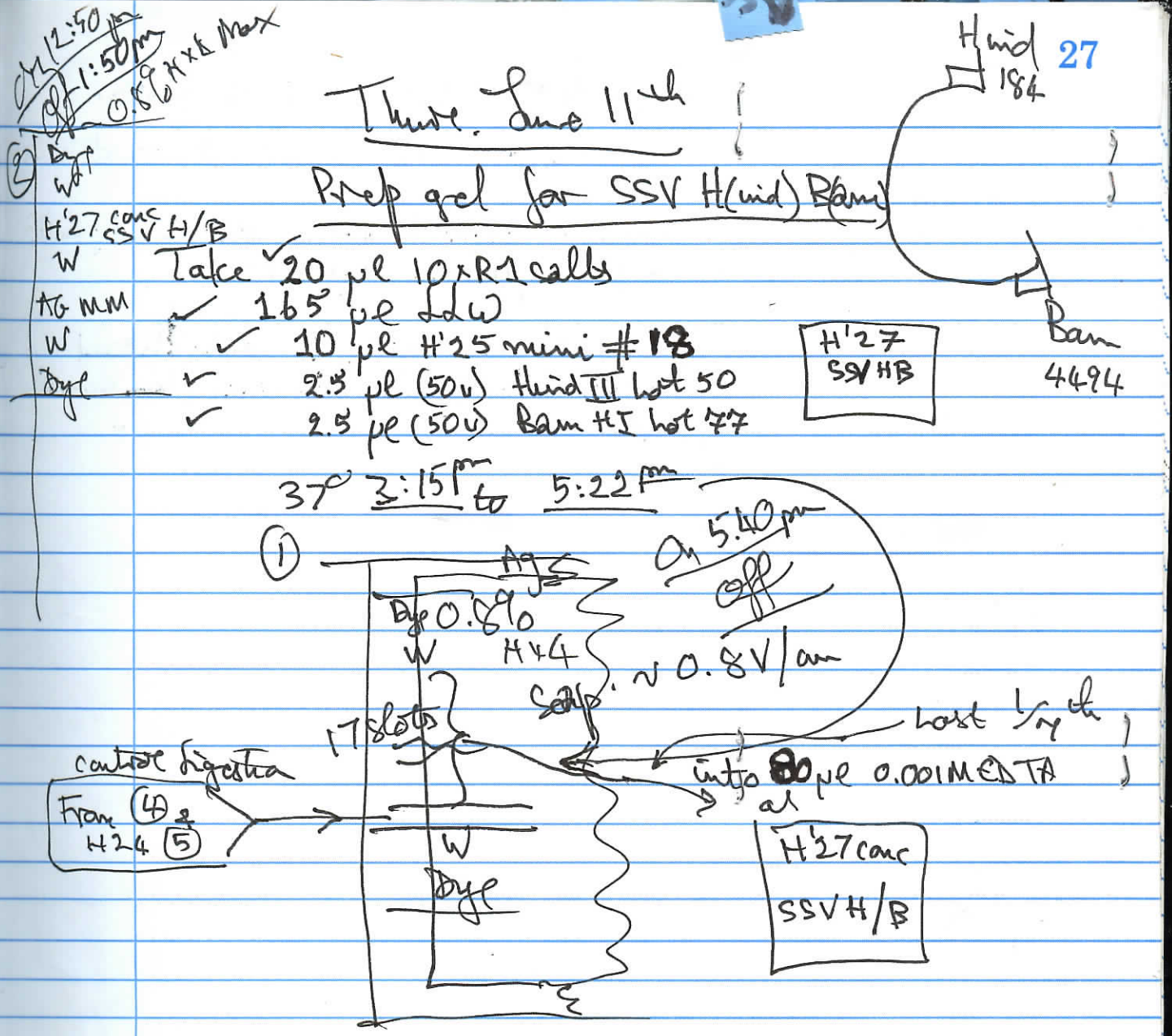
On 12:50 pm
off 1:50 pm
0.8% H×E Max

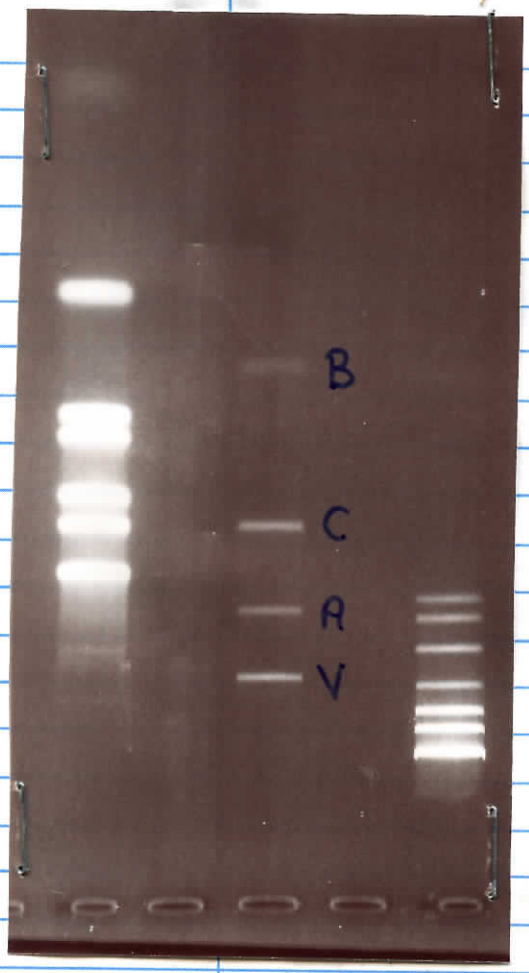
Thurs. June 11th

Prep gel for SSV H(mid) Bam

Take 20 µl 10×R1 salts ✓
165 µl ddW ✓
10 µl H'25 mini #18 ✓
2.5 µl (50u) Hind III hot 50 ✓
2.5 µl (50u) Bam HI hot 77 ✓

37° 3:15 pm to 5:22 pm



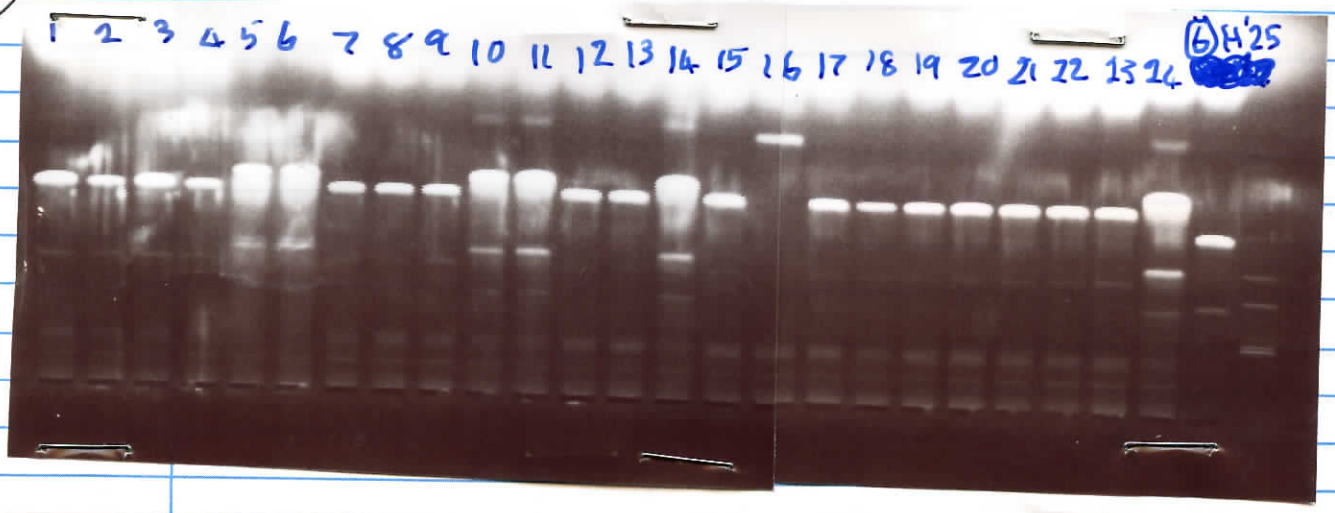


① Conclusion: Proportions are pretty good, but could double A next time.

Simplest diagnostic will be BamH + HindIII; refer to each pair vs. fragments.

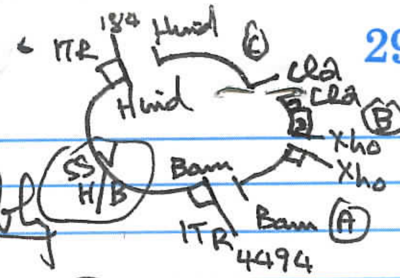
② Conclusion: it was too speedy! Proceed with H'29; it has increased several fold over H'29.

(2)



2) Off 1:10 5.5% HY 1/2 MAX or higher (speedy!)

Thurs. June 12th



First try at SSV. HPRTmini attembly

Dye / Water / (1) (2) ... (23) (24) H'29

H/4 (6) H'25 mini / AGMM / W / Dye — Double this next time

Take 2.5 µl of fragment (4 kb SSV H/B) H'27 conc

✓ 5 µl of fragment (2.2 kb (A)) G'161 conc

✓ 10 µl of fragment (500 bp (B)) H'1 conc

✓ 10 µl of fragment (1.5 kb (C)) H'1 conc

Total 8.0 kb vs. SSV 8.3

(1) 3:30 pm / On 4:00 pm / Off 1.5% HY 10 V/cm

+3 µl 8M NH_4Ac ✓

+75 µl EtOH ✓

Dye / W / AG MM / 2.5 to 12 H'29 Pre / W / ACR MM / W / Dye

✓ Freeze. Cent. Dry & back up into:

{ 10 µl 1x p95 lig^n buffer
+ 0.8 µl T4 ligase lot 67 }

H'29 4F (fragment) LIG^N

✓ 2.5 to 12.5 — H'29 PRE

15° 3:15 pm to 4:20 pm
RT 4:20 pm to 4:50 pm
37° 4:50 pm to 5:20 pm → To cold

2.5 to 12.5 — H'29 POST

Plated 7 pm Sun (GO TO H'31)

12 noon Sat^y → Cold

Rest Sun 4 pm — at H'29^3

Transformation

H'25 5/28 DH5α	80 µl ✗
H'29 4F LIG^N	25 µl ✗
0° 5:20 pm to	5:50 pm ↳ 12:12 pm to 12:55 pm
42°	90 sec

H'29^2 — H'29^3 — ↳ 4:30 pm to 6:45 pm

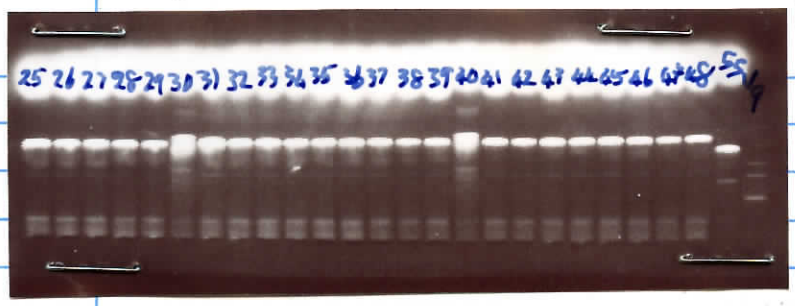
H'29^2 Sat 1 pm — 9 am

Many — Many — (12)

37° Fri 6 pm — 12 noon

Plated 1x AMP N2Y Plat. 20, 5, 20/16, 5/16
(>20) (7) (1) (1) (0)

Mini H'29 (1) — (24)
1x N2Y AMP RT 12:50 pm 37° 5:45 pm



① Conclusion - looks like the impurity in the vector fragment is being preferentially cloned (~6.6 Kb product)

∴ Re-purify SSV H/B, or cut with XcmI or BspEI or EcoNI ↑ or ~~SphI~~ NA

BspEI: Buffer 3, no inact, 100 mM

EcoNI: Buffer 4, 65° 20 min, K+

XcmI: Buffer 2, 65° inact, 50 mM

BspMI: 150 mM NaCl, 65° 20 min

We have EcoNI (1995) Lot 8B 15 U/μl
→ BspMI (1997) lot 30 26 U/μl →

② Conclusion - no improvement. Need to get it pure by

① 10.05am On / 11.10am Off / 0.8 / 0 H'44 Max
Dye / Water / 25 / 26 / : / 47 Rest of / 48 H'25/4F LIGN / 1+14 H'25 mini 6 / Ag MM / W / Dye

Sun June 14th (H'29 contd.)

H'29 (contd)

Picked another 24 minis from H'25² as H'25² ㉕ - ㊽
1x AMP NZY to 37° 4:45pm — 9am

H'25³ 1x AMP NZY Rest, 20, 5, 20/16, 5/16 7pm

Mon June 15th

Resave of vector fragment

Take whole (65 µl) H'27 SSV conc H/B
+ ~~87µl~~ 10x NEB buffer 4
~~[illegible]~~
+ ½ µl (7.5u) EcoNI Lot 8B 15u/µl
37° 3:50pm to 4:50pm
+ 7µl 10xRS + 1.5M NaCl
+ 2µl (4u) BspMI Lot 30 2u/µl
37° 4:56pm to 5:20pm to 6:45pm
+ φOH / HOQ 1x + CIAA 2X → H'31 SSV H/B Conc

② On 5:25pm / Off 6:40 / 0.86 / H'N
Dye / W / H'31 digest / W / Ag MM / W / Dye

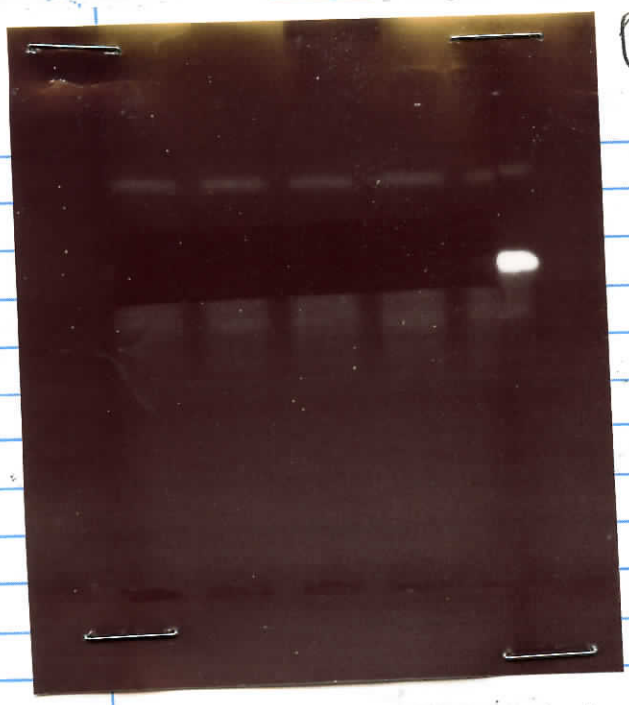
84

Re-assembly of 4E

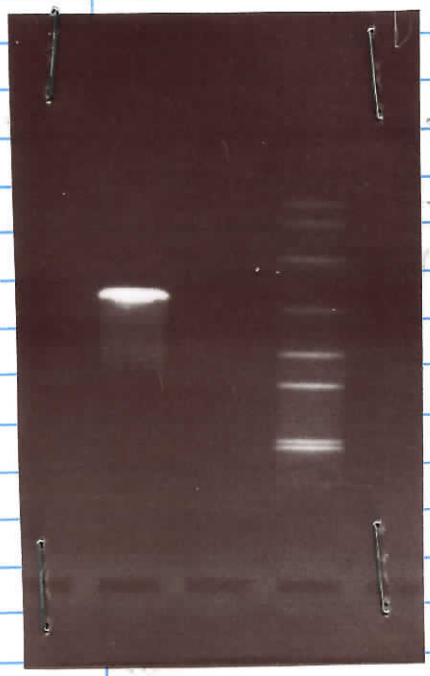
Cla / Cla / Xho / Xho / BamH / 4E / Bam

Take 2.5 µl of fragment SSV H/B H'31 conc 4kb
CANC ~~10~~ µl - - - - A G'161 conc
~~10~~ µl - - - - B H'1 conc
10 µl - - - - C H'1 conc
~~+ 3.5 µl~~ 5M NH₄Ac
+ 90 µl EtOH
Freeze Cent. Dry & back into 10µl 1x PS lig. buffer
2.5µl to 12.5µl + 0.8µl T4 ligase Lot 67
DRE H'31 Cold room — pm

H'31 4F LIGN



① Conclusion: Impurity visible & avoided in cuts.



② Cleaner – proceed

Mon. June 15th

(2) Hind SSV9 H/B II Bam

On 8 pm
off 9 am 0.8 V/cm

Re-purify

(1) 0.8% Ag

Dye
Seap
H'31 SSB H/B conc
4 x H
W
Dye

5 slots

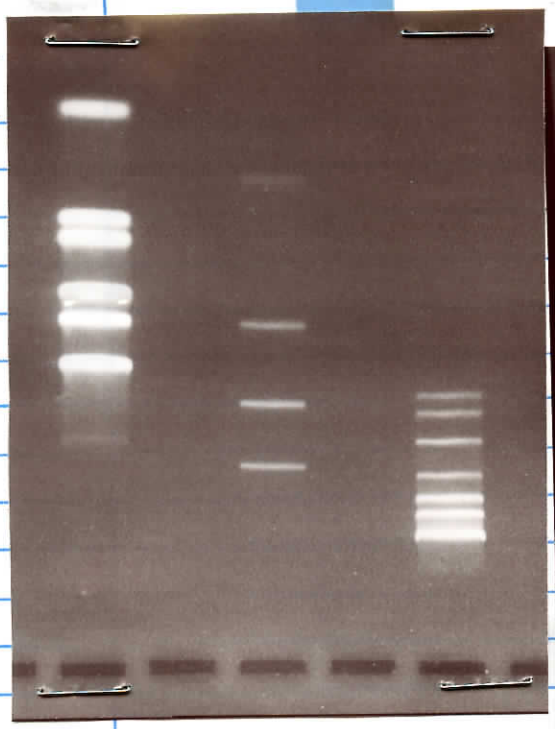
Recovered into 45 µl as

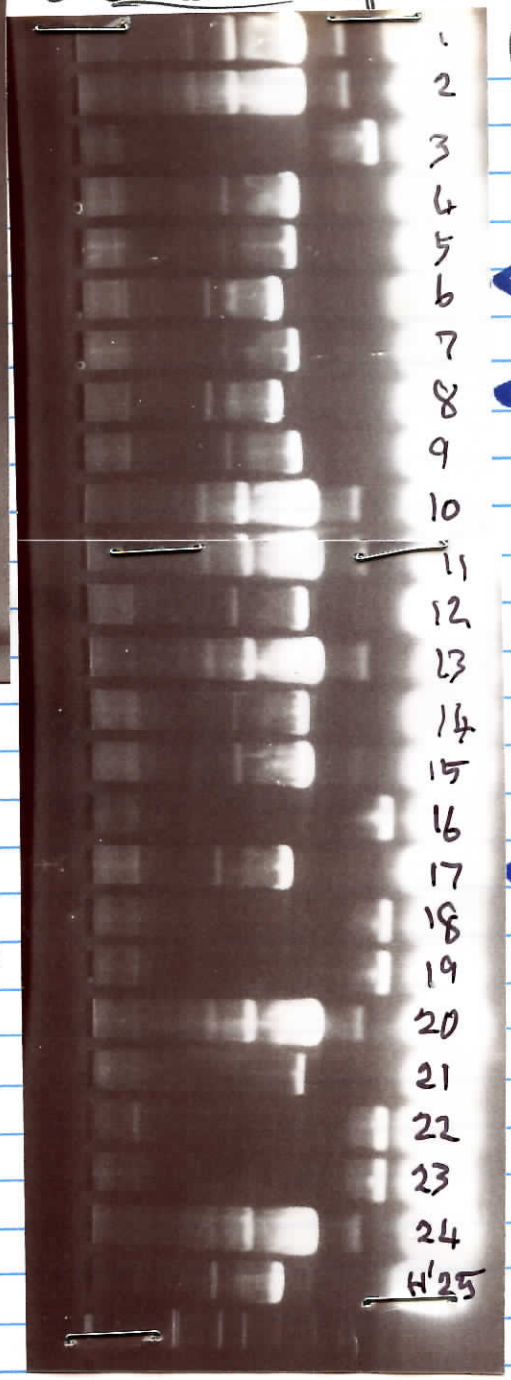
H'33 conc SSV H/B

(2) On 2 pm off 3 pm 0.8% Hx4 Max

Dye
water
H'33 SSV H/B conc
W
Ag MM
W
Dye

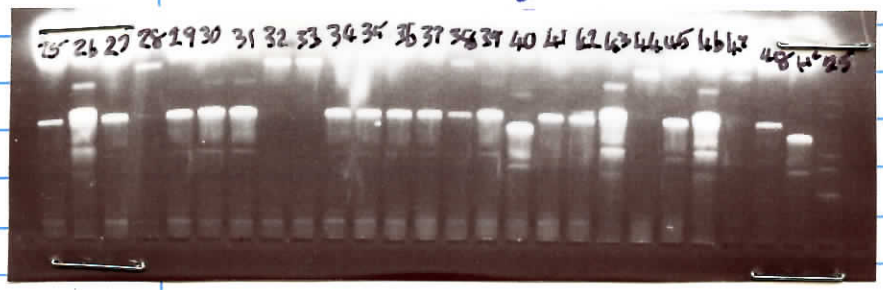
① Conclusion: Proportions are correct





② Conclusion 6, 8 & 17 are possible, 17 less likely

③ Conclusion 40 is possible but less likely



② ON 1:15 0.8%
Dye
W
(1)
(2)
(23)
(24)
H25 #6
AG MM
W
Dye

③ ON 1:24
Dye
W
(25)
(26)
(47)
(48)
H'25 #
AG MM
W
Dye

HX4 M

Vector Insert 1:15 pg 0.8%

Hx4 Max

Tues. June 16th

1) 6:0 pm 6:30pm 1.5% 9 Hx1 10Vl or

Dye
W
1
2
!
23
24
H25 #6
AG MM
W
Dye
1:22pm
9:24pm
Dye
W
25
26
47
48
H'25 #6
AGMM
W
Dye

Dye
W
ACR MM
W
Pre H'35
W
AG MM
W
Dye

Repeat assembly of 4F

Take ✓ 2.5 μl of fragment SSV H/B H'~~33~~ conc.
✓ 10 μl . . . . . . A G'161 conc
✓ 10 μl . . . . . . B H'1 conc
✓ 10 μl . . . . . . C H'1 conc
✓ + 3.6 μl 8 M NH_4Ac
✓ + 90 μl EtOH

~~H'35~~ 4F LIG^N

Freeze Cent - Dry & back into { 10 μl 1x p95 lig buffer
+ 0.8 μl T4 ligase Lot 67

2.5 μl ~~to~~ 12.5 as PRE H'35

Cold room at 3:50 pm
7:45 pm Wed

Back to cold

Wed June 17th
Nancy's day!

Transformation

H'35	DH5α 6/12	✓ 80 μl
		✓ 3.5 μl

0° 1:45 pm to 2:30 pm

42° 90 sec

Plated 1x Amp NZY | Rest, 20, 5, 20/16, 5/16 37° 2:45 pm

Many (19) (2)

Mini H'35 (1) - (24) 1x AMP NZY at 9 am Thurs June 18th
(25) - (48) 9.25 am ~~—~~ 1:40 am

Processed Fri June 19th ~~to completion~~

Gel (2) #6 & #8 are possible, #s 17 ~~[illegible]~~ less likely.
Gel (3) less likely #40 Processed to 50 μl.

Oligo # ____
Date made: ____
LENGTH: 20
% G+C: 50%

5' Oligo Name: H'36 Exon1Blue2654 3'

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

Oligo # ____
Date made: ____
LENGTH: 20
% G+C: 50%

5' Oligo Name: H'36 Exon10 Red3024 3'

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

Printed by: John Hagaman

From: Gregg Semenza <gsemenza@gwgate1.jhmi.jhu.edu>
Date: Thu, 28 May 1998 17:36:29 -0400
To: hage@med.unc.edu

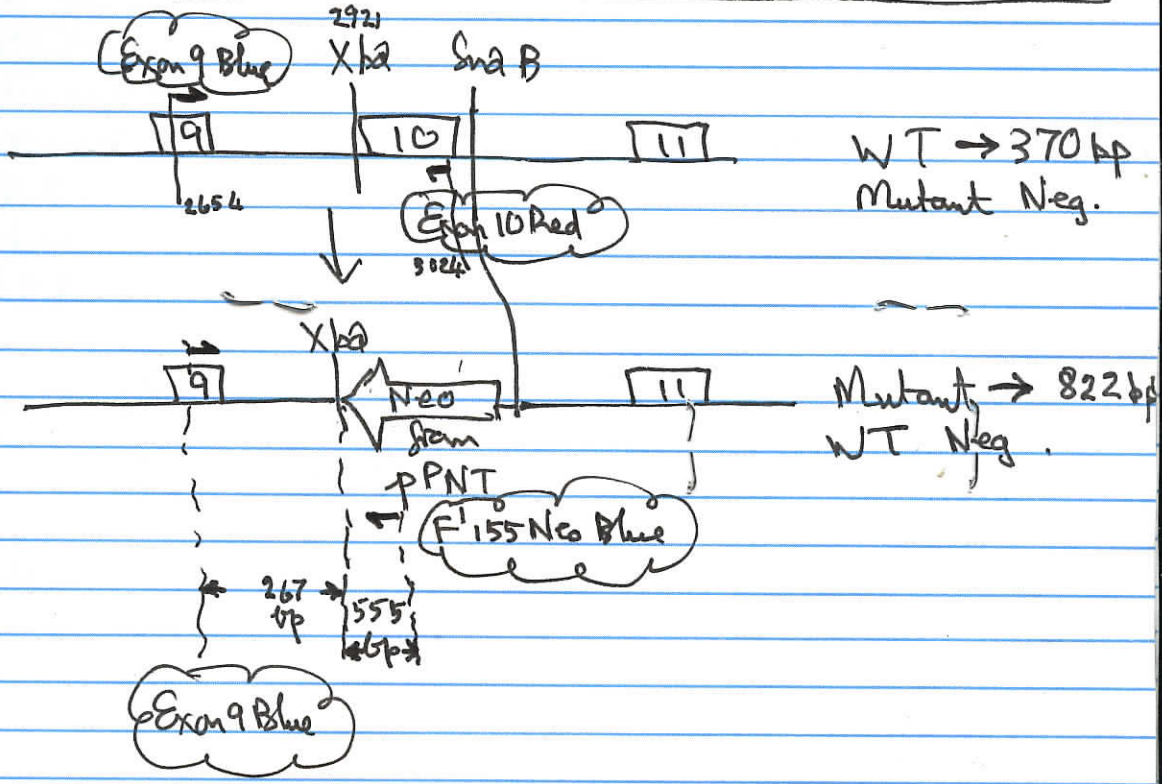
Dear John:
You were previously kind enough to send us Ace (+/-) mice. Can you please tell me the oligonucleotide sequence of the primers, size of the diagnostic PCR product, and amplification conditions that you use for genotyping the mice? We are interesting in making double-heterozygotes with another knockout strain and therefore at least one of the primers must be from the Ace (as opposed to neo) gene. Thank you.
Regards,
Gregg Semenza

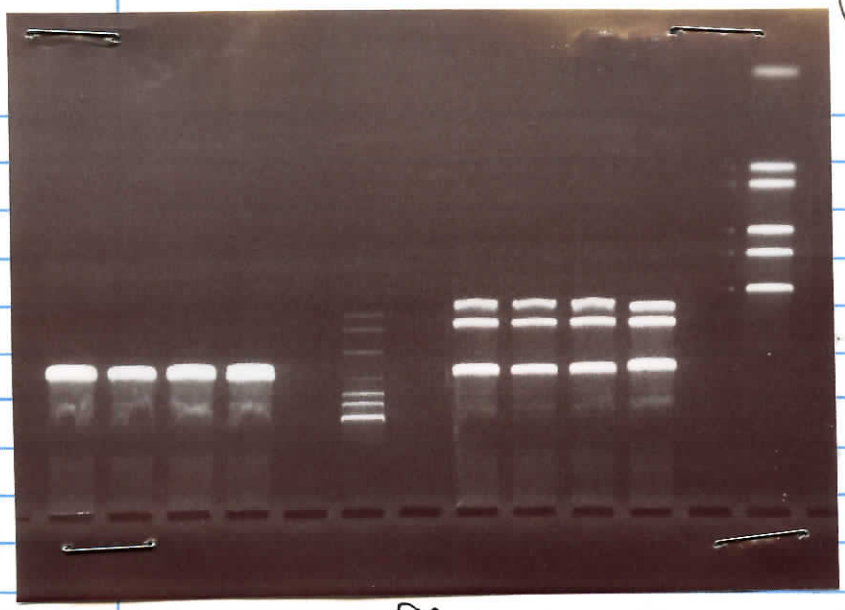
Gregg L. Semenza, M.D., Ph.D.
Associate Professor of Pediatrics
Institute of Genetic Medicine
The Johns Hopkins University School of Medicine

Johns Hopkins Hospital, CMSC-1004
600 North Wolfe Street
Baltimore, MD 21287-3914
Tel 410-955-1619
FAX 410-955-0484

Thurs. June 18th

PCR primers for ACE Exon 10 → Neo

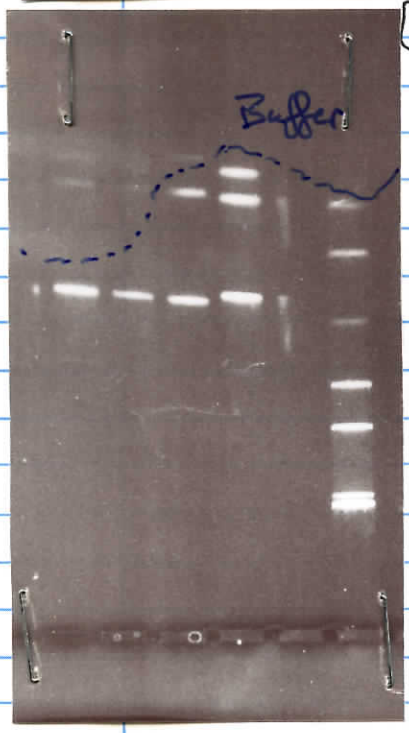




(1) Conclusion

Wrong gel! but #40 is looking good, others are still possible. Only one Xho site.

Run residues in 0.8% — but repeat with 1.0% & ± Cla.



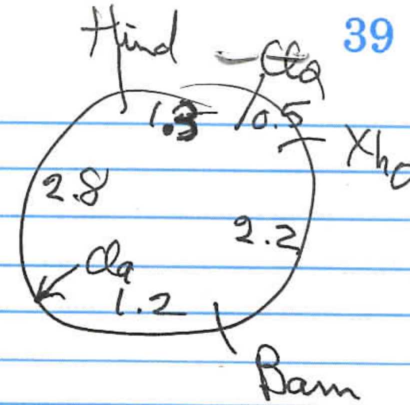
(2) conclusion 6, 8 & 17 differ from 40 — can't tell which is correct.

① ~~4:15pm~~ 5:35pm Agx1
~~Off~~ 1.5% + 2 μl /ml [10] RNAse

Dye
W
6, 8, 17, 40 } H/B
W
Ag MM
W
6, 8, 17, 40 } H/B/X
W
Acryl MM
W
Dye

Fri June 19th

Tests of candidates



Hind, Bam ± Xho is a good ~~first~~ test

Take ✓ 20 μl 10x R1 salts
✓ 180 μl ddw
✓ 2 μl (40 u) Hind III Lot 50 (20u/μl)
✓ 2 μl (u) BamHI Lot 77 (20u/μl)
✓ 100 μl | 100 μl + 1 μl (24 u) XhoI Lot 35 (20u/μl)

✓✓✓ 4x 20 μl aliquots | ✓✓✓ 4x 20 μl aliquots
Expect 4kb & 3.2kb | 4kb, 2.2kb, & 2kb

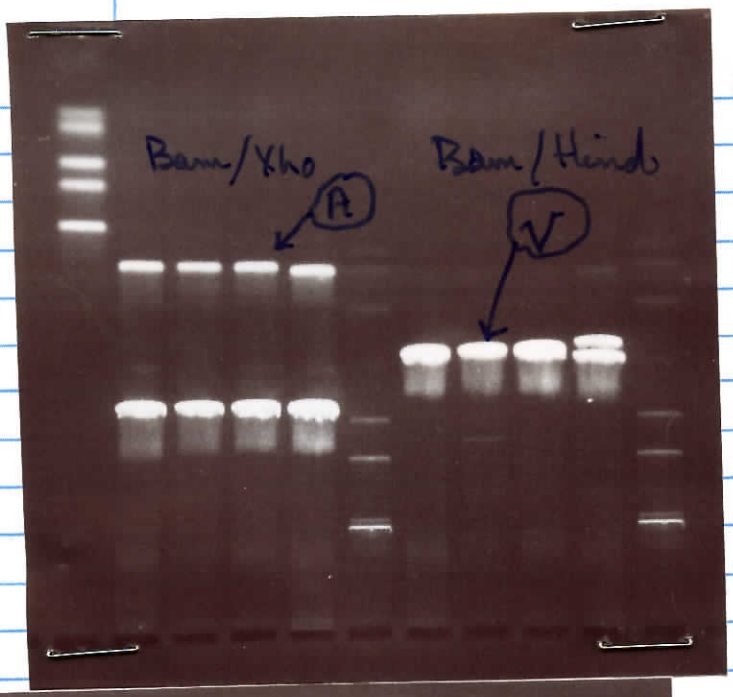
Each + 1 μl of H'35 minis # 6, 8, ~~17~~ & 40

37° 3:25pm → 4:45 pm

② ~~On 6:30pm~~ 4:20pm Ag x4
~~Off~~ 0.8% + RNAse
4V/cm

Dye
W
6, 8, 17, 40 } H/B/X
W
AG MM
W
Dye

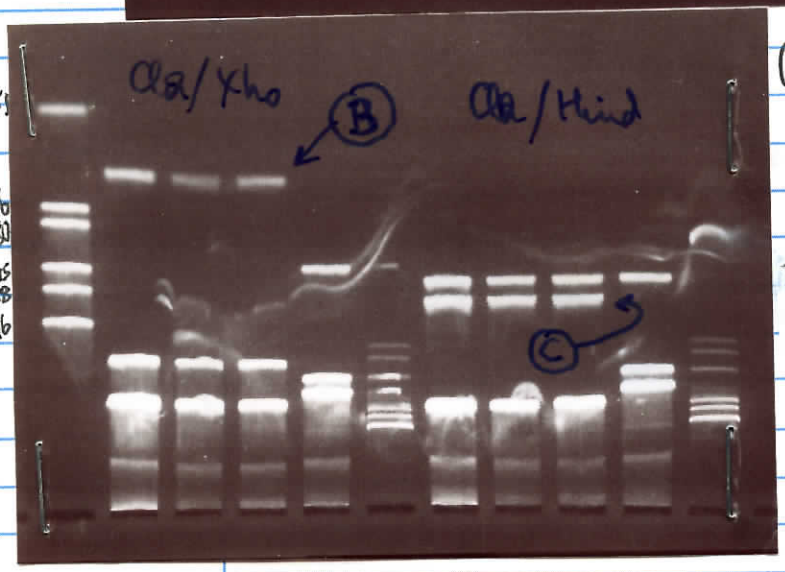
H'39 6, 8, 17 & 40 H/B	H'39 6, 8, 17 & 40 H/B/X



Needs better gel

(1) Conclusion (A) in (6), (8) & (17) / 5.8 is O.K.
– not in (40)

(V) c'd be in all 4, but see H'38 gel 2. / 4.0 is also O.K.



(2) Conclusion (B) in (6), (8) & (17)
– not in (40)
(C) in (6), (8) & (17)
– not in (40)

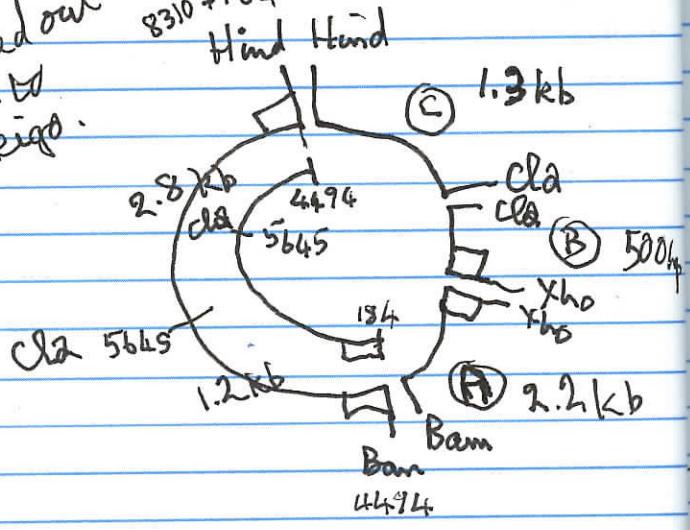
Also the assignment of Hind & Bam in vector was incorrect.

Overall conclusion

H'35 (6) (8) & (17) are correct! Streaked out & 17 to seqn.

but re-run all on long & careful 0.8% gel

and the Cla again on a 1.5%

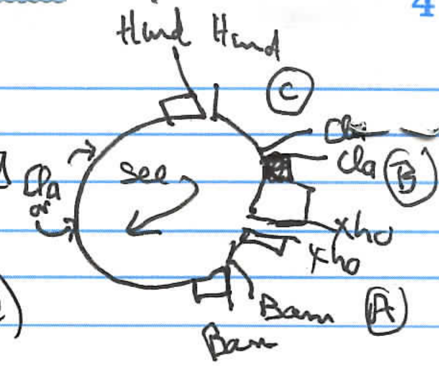


Well: 0.8% H×4 2µl RNAse 2/3 Max

Dye
Water — Acry MM
6, 8, 17, 40 — B/X
Ag MM
6, 8, 17, 40 — B/H
Ag MM
W
Dye

Sat. June 20th

More candidate tests



Take ✓ 34 µl NEB buffer 4 (KCl) 10X
✓ 34 µl 10X BSA
✓ 255 µl ddw
✓ 17 µl (80 u) Cla I Lot 33 (5u/µl) 5u/digest
✓ 80 µl ....

H'35

+4 µl mm → 6 8 17 40 37° 11:09 am – 1:38 pm

(8 by mistake)

✓ +8 µl 10×R1 salts ....

50 / 50

+½ µl (10u) ✓

Xho Hind Xho Hind Xho Hind Xho Hind

Xho I Lot 35 20u/µl
Hind III Lot 50 20u/µl

H'41 6 (CX), H'41 6 (CH), H'41 8 (CX), H'41 8 (CH), H'41 17 (CX), H'41 17 (CH), H'41 40 (CX), H'41 40 (CH)

37° 1:53 pm – 4:50 to cold

CX: B 0.5 — 3.4, 4.1 — OR 2.5 + 5.0
CH: 1.3 C — 3.9, 2.8 — OR 1.2 + 5.5

Run in 1.5% gel + RNAse

1.5% H×1 RNAse o/n

Dye
W
ACR
6, 8, 17, 40 — C/X
+ 2 µl H'35 mini
Ag
6, 8, 17, 40 — C/H
Ag
W
Dye

Take ✓ 34 µl 10×R1 salts
✓ 302 µl ddw
✓ 4.4 µl Bam HI Lot 77 (20u/µl)

✓ 170 µl ✓ 170 µl

✓ +2 µl Xho Lot 35 | +2 µl Hind III Lot 50 (20u/µl) ✓

✓ 40 µl aliquots ✓ 40 µl aliquots

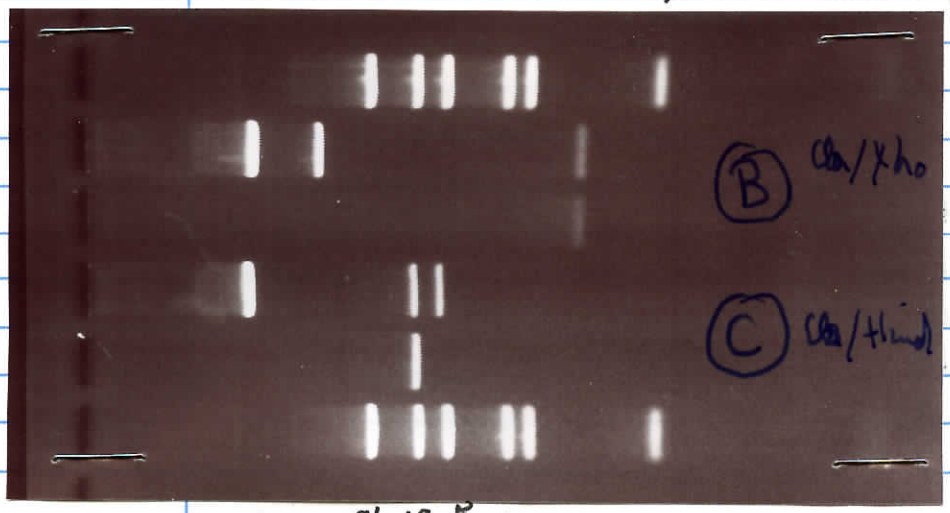
6 B/X, 8 B/X, 17 B/X, 40 B/X | 6 B/H, 8 B/H, 17 B/H, 40 B/H

BX: 2.2 A, 5.8
BH: 4.0, 4.0 V

37° 11:28 am to 1:20 pm to cold

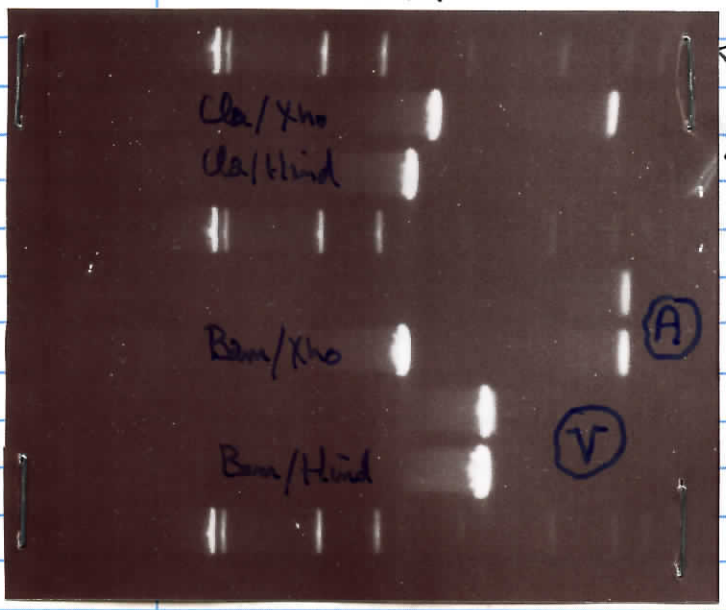
Run in 0.8% gel + RNAse

Cla/HindIII 7.7kb
↓ 696 351



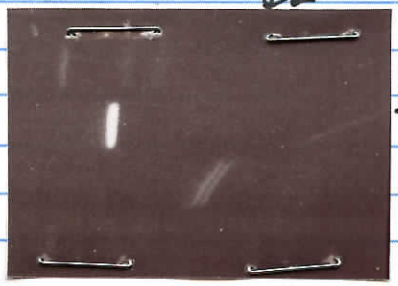
① Conclusion
B & C are fine.
0.5kb 1.3kb
+ auxiliaries

1696 1268 1078 kb
Cla/HindIII 5.5kb 5.0kb Cla/Xho 2.4kb Cla/Xho



0.5 was visible

② Conclusion
2.2kb A & V 4kb are fine
+ auxiliaries
but effect 1.2 & 1.3 kb



#17 is fully confirmed!

—will call this H35 HPRTA 17

Note that Cla polarity (i.e. Xba–Xho orientation) is inverted relative to Samulski's SSB 9 map

Judge confirms that SSB 9 & 16 have opposing orientations & may be inverted

Sunday June 21st Checks

Check on #17 H'35

Xho (A) 2.2 Bam

(B) Cla 0.5 Xho

1 2 3

(C) Hind 1.3 Cla

SSB H/B Vector Hind 4.0 Bam

On 1:30pm Off 1:30pm 1.5% H×1 RNAse 1V/cm

1

Dye

Water

ACR MM

Fragment (B) conc H'1 undiluted

(17) Cla/Xho H'39 Diluted 10+10

Fragment (C) conc H'1 undiluted

(17) Cla/Hind H'39 Diluted 10+10

ACR MM

W

Dye

On 1:24pm Off 5pm 0.8% H×4 RNAse 4 V/cm V=3 at 2:10pm

2

Dye

W

AG MM

(17) Cla/Xho ~~Diluted~~ 10+10 H'39

(17) Cla/Hind Diluted 10+10 H'39

AG MM

Fragment (A) conc G'161

(17) Bam/Xho ~~Diluted~~ 10+10 H'39

Fragment (V) SSB H/B conc H'33

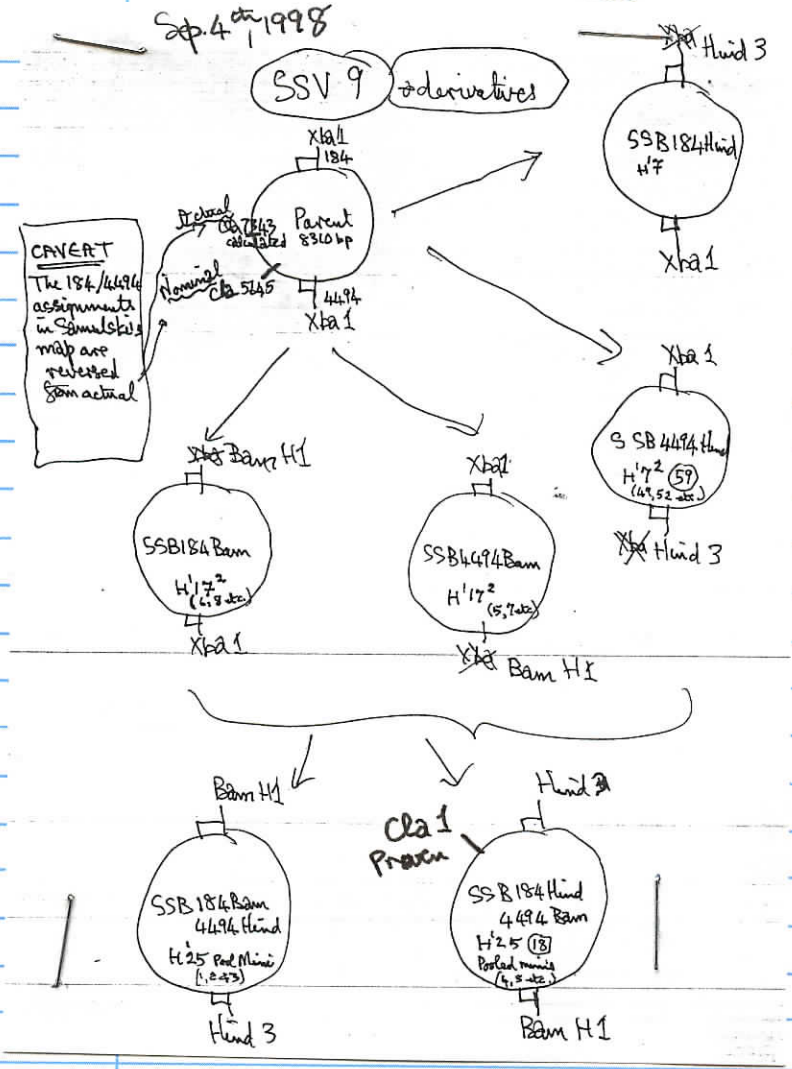
(17) [redacted] Bam/Hind ~~Diluted~~ 10+10 H'39

MM AG

W

Dye

Sep. 4th, 1998

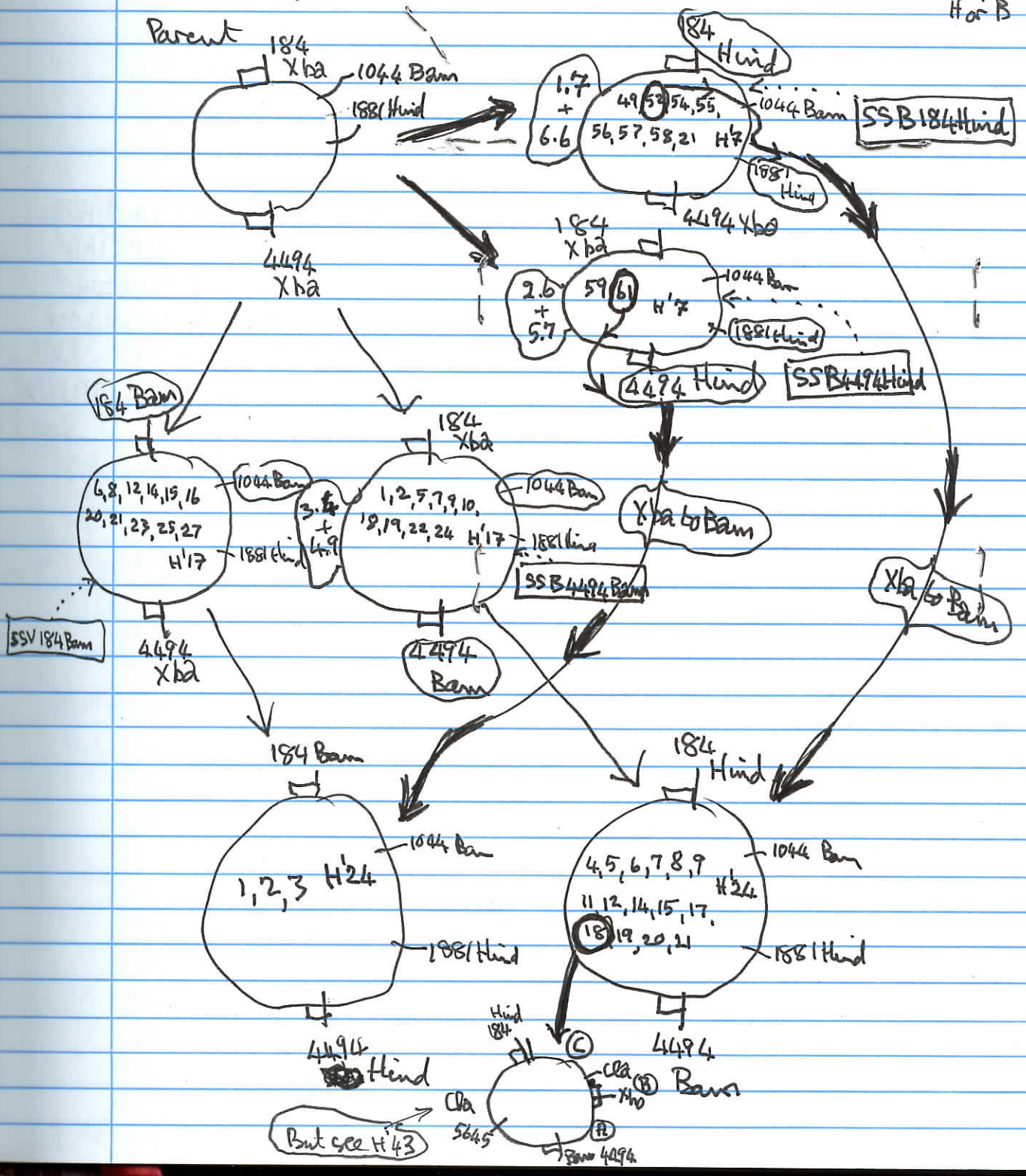


SSV 18

Mon. June 22nd

SSB-9 derivatives tidy up

Following forms are desired:

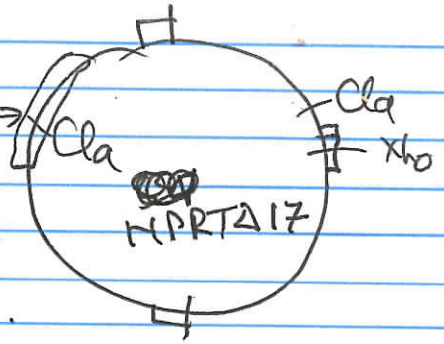


① Good series. Pooled 2, 4, 8 + 16.

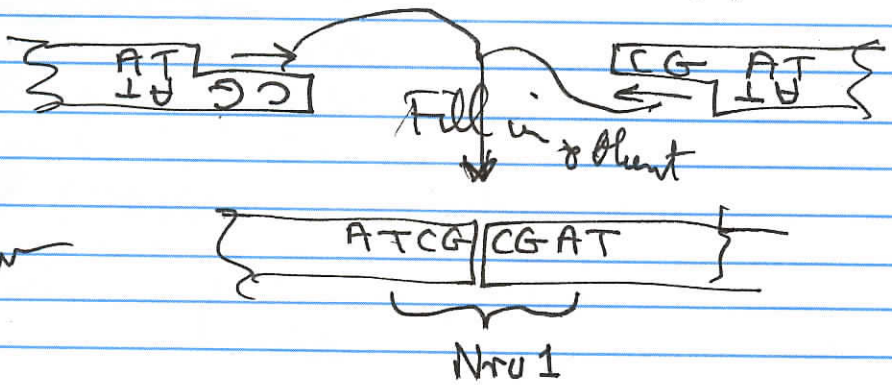
② Good cut. 1x Φ/HOQ
2 x CIAA
2.5 x EtOH
Back into 80 µl 0.001M EDTA
as H47S (single) Cut) Ch Conc
Qd on 4'53
(and Blunt ended)

Sun June 28th

Single Cla I cut for Removal of Cla I in H'35 HPRTΔ17 in F1 regia



The F1 regia of SSV9 is irrelevant & can be removed.
∴ change extra Cla I to Cla to facilitate later changes in HPRTΔ17 (e.g. change of promoter).



Take 20 µl (~10 µg) H'35 mini #17
60 µl 10× NEB Buffer 4
360 µl 10× BSA
460 µl dH2O
2 µl Cla I Lot 33' (5 u/µl)

RX

Preheat to 37°C

80 µl to 8 µl 1.5M NaCl+10×RS → to 80°C for 5 min

18 µl

Pre "0" 1 2 4 8 16 32

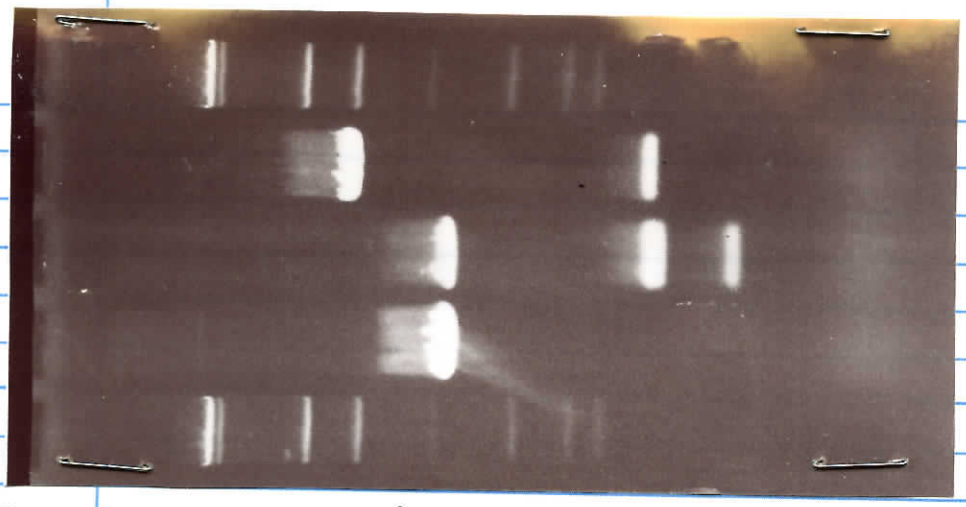
Run gel. Pool. Prep. gel. Ppt. Fill in. Ligate.

Alternative is to excise F1 & ligate. See H'57

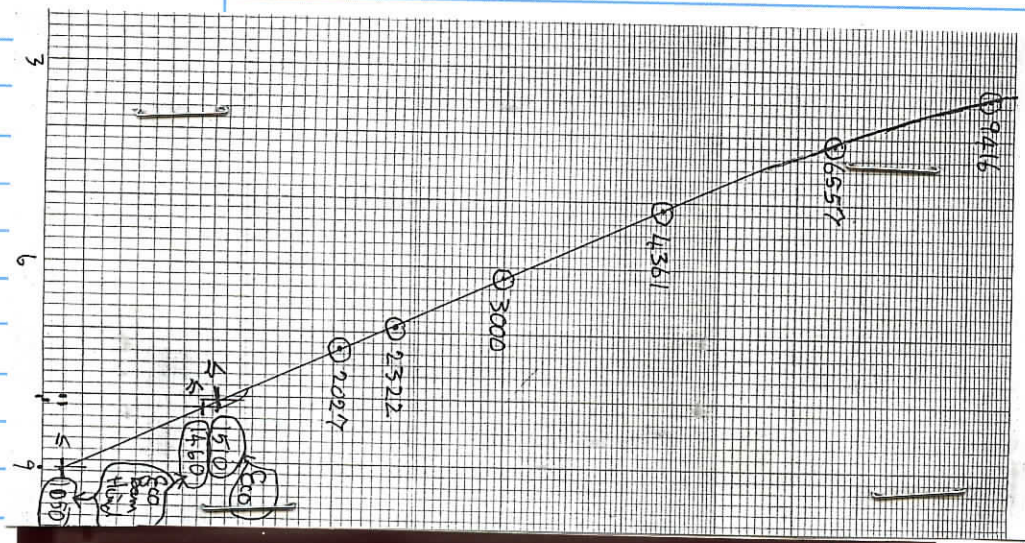
Pooled samples 2, 4, 8 & 16 to vol. of 180 µl
Added 12 µl 2.5mM 4dNTP's to give ~100 µM of each

+10 µl (20 units) Klenow Polymerase 2u/µl
RT (26°C) 6:15 pm — 6:45 pm
Not 11-50/31 Jan 00

H'47 Klenow



① Conclusion: EcoRI looks good, but measured size ~ but c'd be extrapolation. of small frag^t is ~1.5 kb, not 1.3 kb!. No sign that Eco cuts in the 4kb HPRTΔ part, but the expected 2.5 kb band is missing → is replaced by ~1.46kb + ~1.050 kb i.e. an extra Bam or Hind site is present. Reversing Hind → Bam will not restore this. Proceed with ligation → think. Run frag^{ts} on 1.5% fast gel.

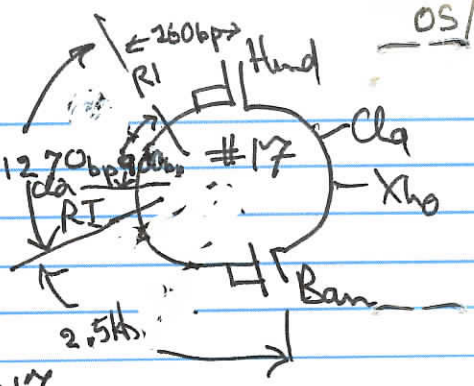


② Conclusion: Get 2 confirms that something is wrong!

OS/RWS 49 day!

Tuesday June 23rd

Rough test of RI excisa



Take ✓ 5 µl H'35 mini #17
✓ 10 µl 10× Eco RI buffer
✓ 103 µl dd w

80 µl
✓ + 1 µl Eco RI (20u/µl) lot 27
20 µl ✓

40 µl
✓ + ½ µl lot 25 (20u/µl)
½ µl
20 µl { Bam HI + Hind III lot 50 (20u/µl)

H49	RI
	17

Expect 1.3 kb + 6.7 kb if no other cuts

RI + Bam + 17 + Hind

Expect 1.3 + 2.5 + 4.0 ← Will not change if no other cuts. 260bp

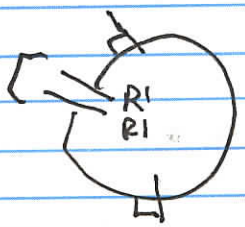
Bam + Hind 17

Expect 4.0 kb + 4.0

37° 9:15 am
10:30 am
11 am
↓
1 pm
70° for 25 min
75°

Tues. June 23rd

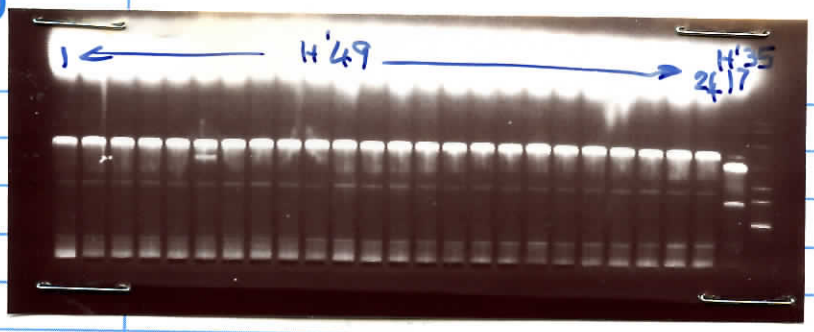
Eco RI digest ligation



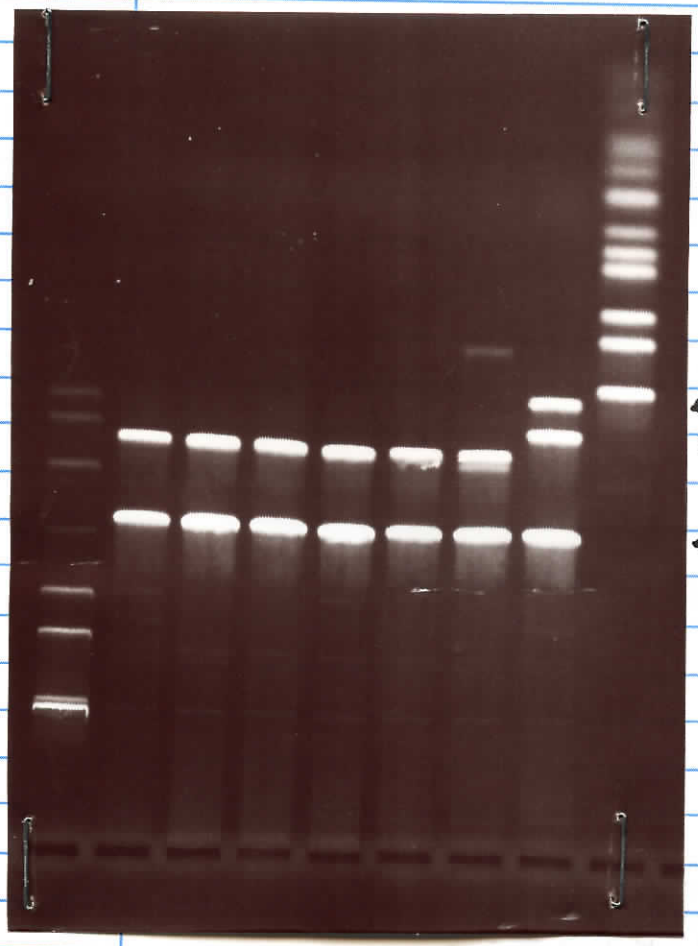
Take ✓ 2 µl H'49 RI 17
+ 16 µl dd w
+ 2 µl p95 lig buffer 10×
+ 1 µl T4 DNA ligase lot 67

10 + 5
Pre lig H'49

15° 2:15 pm to 4:40 pm
37° 4:40 to 5:45 to cold



① Conclusion - all but an impurity in #6 are correct.



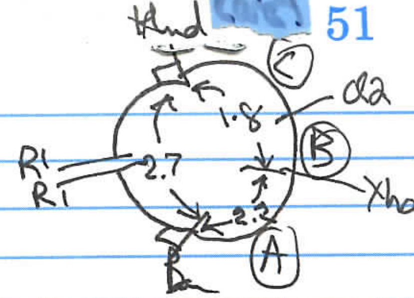
② Conclusion: None are any good - the H'48 gels are correct - there must be an Eco site in the HPRT Δ region

Go back to original H'47 plan.

←1.8
←2.2
←4.0
} as expected for #17

Tues. June 23rd

RI excision HPRTΔ



① On 10 am / 11:30am Off 0.8% HX4
Dye
W
①
②
⋮
㉓
㉔
H'35 #17 mini HX4
AGMM
W 1X AmpNZY
Dye

Transformation

H'35	DH5α	80µl ✓
	H'49 LIGN	3µl
	0° 5:48pm to 6:[illegible]15pm	
	90 sec 42°	
	Plated Rest	20, 5, 20/16, 5/16 37° 6:25pm → ~9am
	Picked ①–㉔ H'49 minis	37° 5pm Wed. (Many!)

1:13 / 2:30 On HX4 RNAse MAX 0.8%
② Dye
Water
AGMM
1–6 H'51 minis H/B/X
H'35 #17
ACR MM
W
Dye

Minis ①–⑥ processed Blind to 50µl.

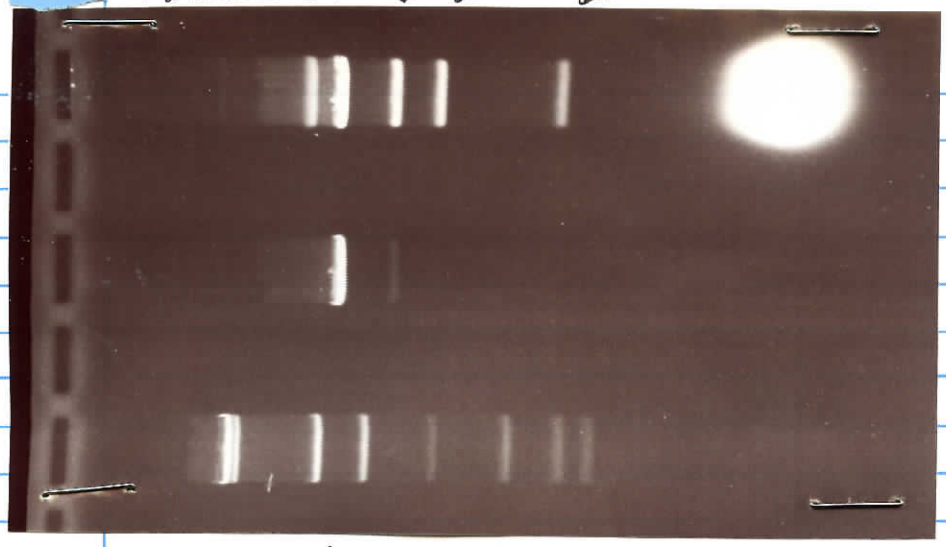
Take ✓ 16µl 10X RI
✓ + 140µl ddw
✓ + 2µl (40u) HindIII Lot 50 20u/µl
✓ + 2µl (40u) Bam HI Lot 77 20u/µl
✓ + 2µl (40u) Xho I Lot 35 20u/µl

20µl aliquots + 1µl minis H'51 ①–⑥ & H'35 Mini ⑰
37° 11:40am → ~1.0pm

Expect:
H'35 #17 ~~4kb~~ (vector) ← I.TKb
2.2 kb Fragment Ⓐ ← ditto
1.8 kb Fragment Ⓑ+Ⓒ ← ditto
Correct Eco w'd be →

Going back to [illegible] Aya Jakovits paper shows the mistake – there is an Eco RI site 1.15kb 3' to exon 3 XhoI site – it will generate the 1.05kb Eco 5' human Bam [illegible] seen on H'48 gel.

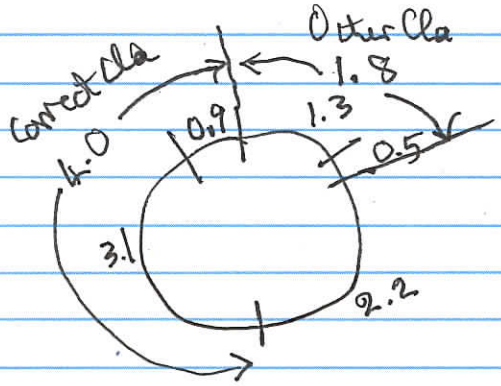
Nicked ↓ Double ← Supercoil — Double cut



↑ Single cut

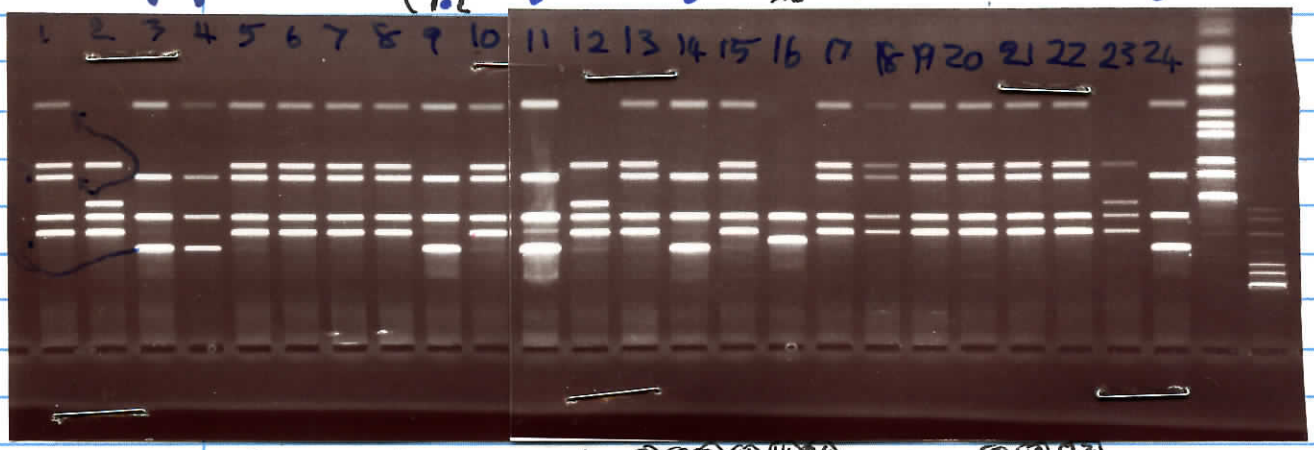
① Conclusion

Good yield, the trace of double cut won't hurt.



Unique

0.5		0.5
0.4	0.9	1.3
1.3	1.8	2.2
2.2	2.7	
3.1	3.1	4.0



② Conclusion 6 are correct, 3 are other Cla site, 14 are unmodified. 1 (#16) is double cut, no mix.

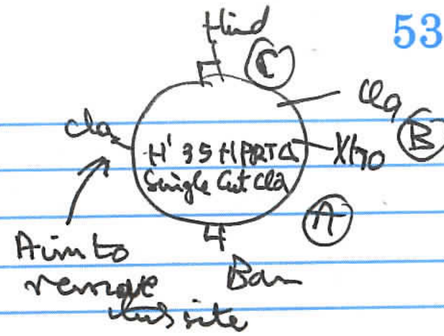
(H'53 contd.) 37° 1:35 to 2:30 pm Gel run 2:50 → 3:20 pm

Streaked out H'53 #9 for Seigo at 37° 5:45 pm Wed.

H'53 HPRTΔ9

DW/MID.

Mon. June 29th



H35 [scribbled out] HPRTΔ Single Cut Cla

① 0.8% 9HX4, 11:30a 250mA
② 1:0p
Dye
Water
H'47 Klenow (pore)
② 0.9% HX1, 1.5% Rose, 2:00pm, 3:20
W
H'47 SC Cla conc (A) → Added to (B)
W
AGMM
W
Dye
Dye, W, ①, ②

Ligation

Take ✓ 10 μl H'47 SC Cla (A) (Cut & Blunted)
✓ 6 μl ddW
✓ 2 μl 10x p95 lig^n buffer
✓ 2 μl T4 ligase (Biolabs) lot 67'

H'53 lig^n

H'53 Cla/Xho/Bam/Hind

37° 1:30pm to 2:55 pm → to cold.

㉓ ㉔ W AGMM W Dye

Transformation

H'35	DH5α	80 μl
	H'53 lig^n	6 μl
	0° 3pm	to 4:10pm
	42°	90 sec

H'53 Cla 1

Many ㉓ ④

Plated on 1x Amp NZY Rest 20, 5, 20/16, 5/16.

37° 4:20pm – 9:40am

Tues. June 30th
Picked minis H'53 ①–㉔ RT at 10am 37° 3:50pm to 9:15am

Aliquoted to 50 μl. Wed July 1st

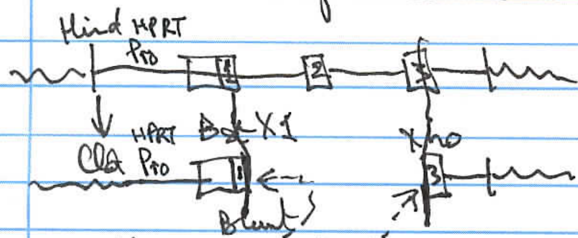
Take ✓ 52 μl 10x NEB buffer 4
✓ +52 μl 10x BSA
✓ +390 μl ddw
✓ +26 μl Cla I lot 33 (5u/μl)

52 μl 10x R I buffer
✓ 453 μl ddw
5 μl Xho I lot 35
5 μl Bam HI lot 77
5 μl Hind III lot 50

✓ 29 μl aliquots + 2 μl ✓ mini 37° 11:30am to 1:20pm, then add 20 μl

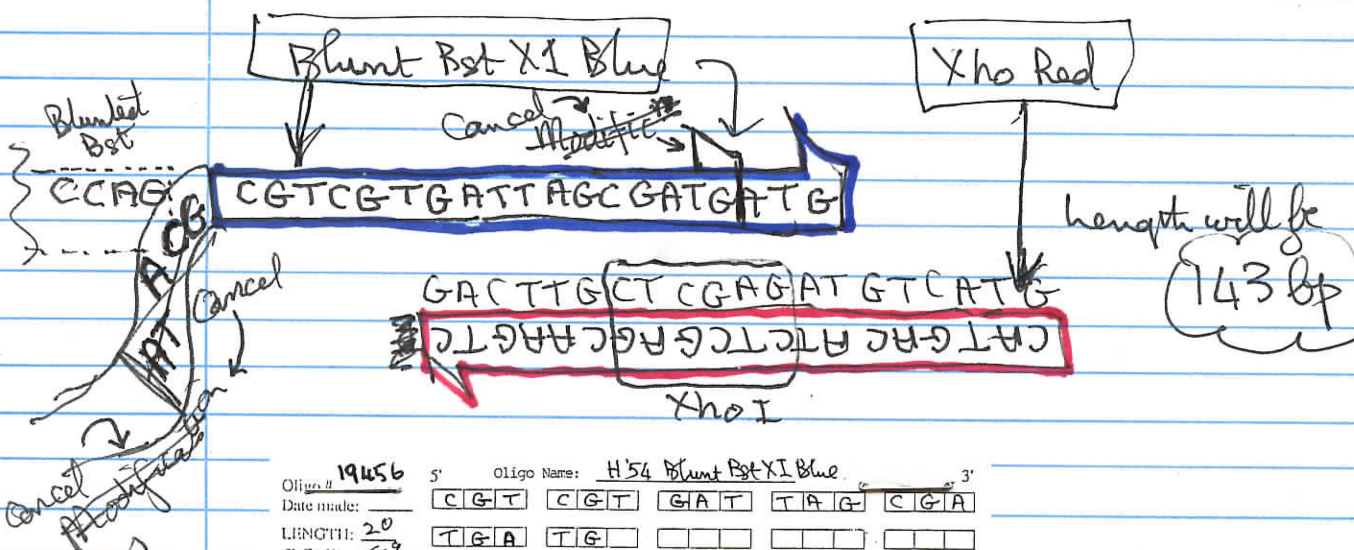
not critical — and XhoI

Steps are :—
Check Hind III, Bst XI (and Cla I) sites in DWM 110.
If acceptable, change Hind III in DNM 110 to non-GATC Cla I.
Cut the product with Bst XI & Blunt with T4 pol.
Cut again with Xho I :—



Using pnNI make PCR product & cut with Xho to give

Primers needed are :—



Oligo # 19456	5' Oligo Name: H'54 Blunt Bst XI Blue				3'
Date made:	CGT	CGT	GAT	TAG	CGA
LENGTH: 20 / % G+C: 50%	TGA	TG			

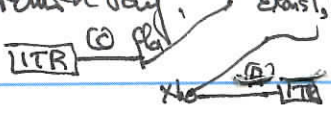
Oligo # 19457	5' Oligo Name: H'54 Xho Red				3'
Date made:	CAT	GAC	ATC	TCG	AGC
LENGTH: 20 / % G+C: 50%	AAG	TC			
19458 H'54 Hind to Cla	AGC	TGC	ATC	GAT	GC

Poss. Improvement : Put a restriction site on end on the PCR product to get clean Blunt end with phosphates. But no enzyme exists for ACG|CGT. See H'60.
XhoI end

1 → 2 → 3

cut

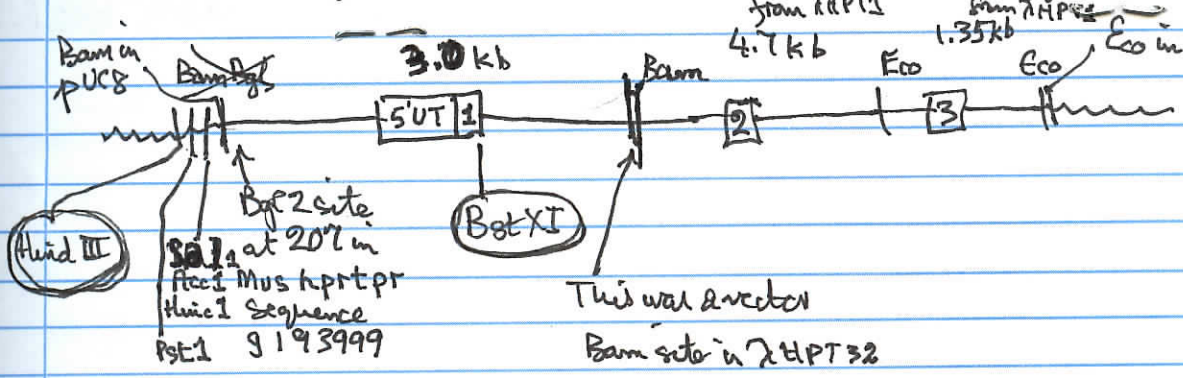
NM reduction day.



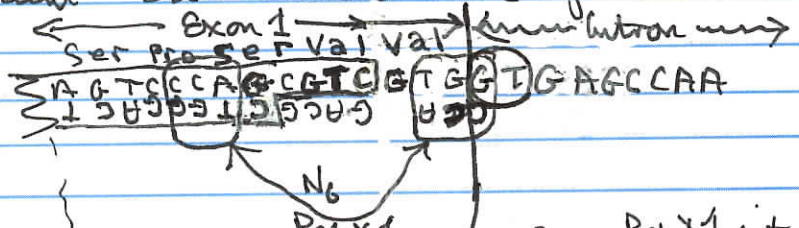
Sat - July 4th

HPRT promoter hamst - recomb. construct

pDWM110 is an HPRT minigene under the control of 637 bp of the natural HPRT promoter. Its history is from 3 fragments

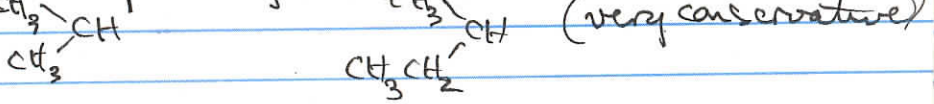


Sequence of mus hprt promoter exon 1 shows that exon 1 has a convenient Bst X 1 site at 3' end of exon 1 :—



The cDNA is

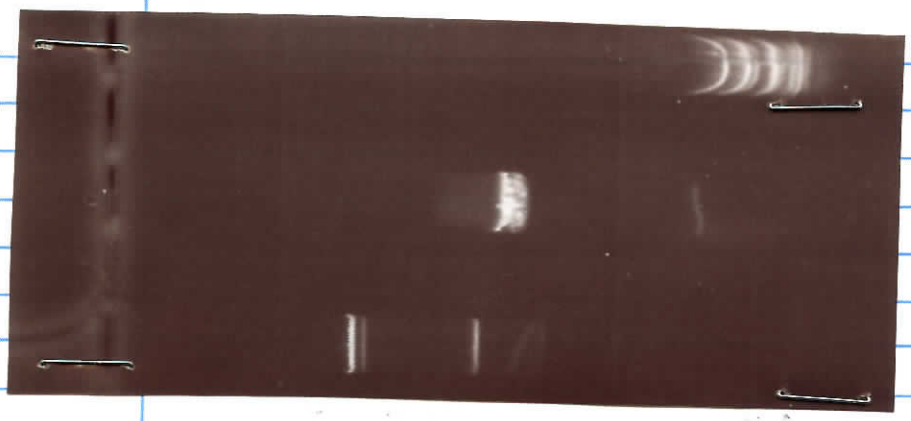
Can therefore change Hind to Cla and excise a Cla - Bst XI piece, and use PCR with Pvu NI and get Bst X1 ---- Xho. This will work but with a Val in place of Ile



Alternatively I can cut pDWM110 with Bst X1 blunt & with Xho & fill in with a PCR Blunt ---- Xho.

This is not appreciably more difficult, & should give certain product

cont'd



(1) Conclusion:
Buffer exhausted
but 2 or 3 thick
bands anyway!
Do partial ↓
use EcoR1 buffer

Ma. July 6th

Quick try at HindIII on pDWM110

Take 1 µl (~1µg) Seigo's DWM 110 F66 (5)
+ 8µl ddw
+ 1µl 10× HindIII buffer H3
+ ½ µl (10u) HindIII Lot 50 (20u/µl)
37° 4:25pm to 5:30pm

H'57 HindIII 10

Cancelled

12 µl ddw 2µl p95 10× ligase buffer
4µl H'57 HindIII 10
2µl H'54 HindIII to Cla oligo
2µl T4 ligase.
37° 5:32 to 8:30pm → to cold

H'57 Lig^N

Transformation

DH5α	80µl
H'57 lig^n	4µl
0°C 8:30	to —
42°	90sec.

Plated 1× Amp NZY Rest, 20, 5, 2^0/16, 5/16
37° —

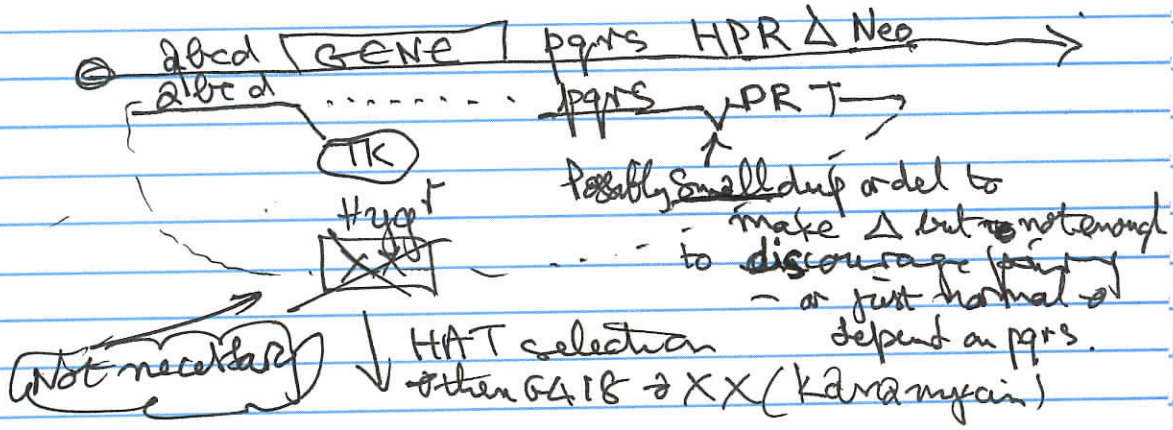
5/28 H'25

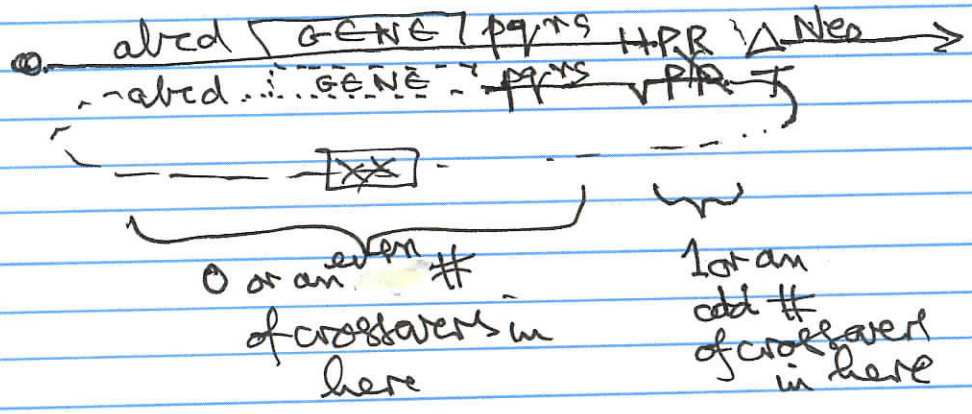
Cancelled

Tues. July 7th

Some thoughts on gap repair (modified July 28th)

Possible use of HPRTΔ × ΔHPRT to select in long gap repair for Noburyuki.





Talking with Noburyuki suggests that first try might be with the Alan Bradley's method if we can get the vectors.

Oligo # ____
Date made: ____
LENGTH: 28
% G+C: 46%

5' Oligo Name: H'60 EARPRI(primary) 3'

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

Oligo # ____
Date made: ____
LENGTH: 21
% G+C: 48%

5' Oligo Name: H'60 EARSEC(ordary) 3'

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

each + 500µl 0.001M EDTA
1X CIAA

H'60 EARPRI

H'60 EARSEC

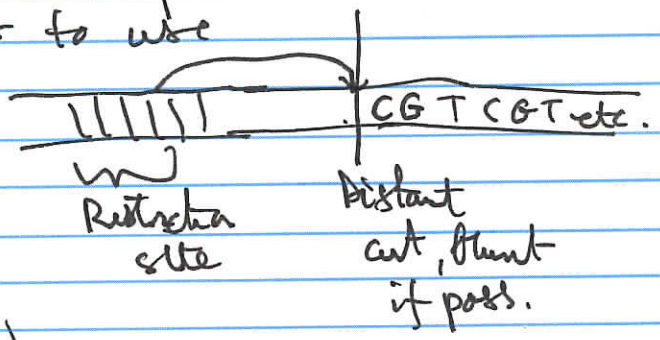
Back from Australia & Taiwan

CATA

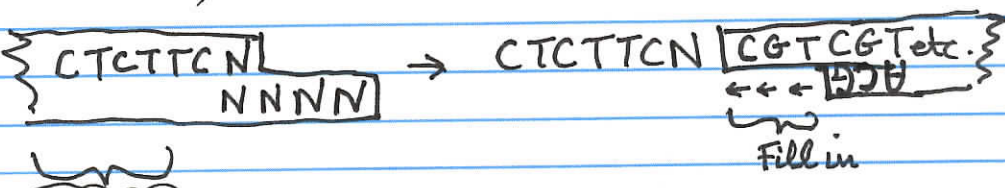
Sat. July 25th

Some thoughts on HPRT promoter (H'54)

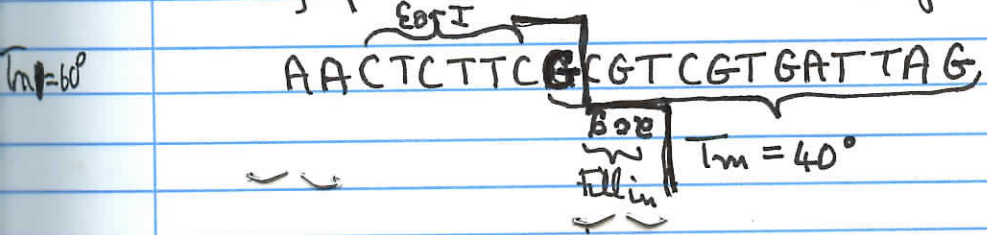
Blunt Bst X1 Pstn is a little dangerous because PCR is not precise on ends. Probably better to use



Possible [redacted] (also Marshall) :—



Primer will be as follows with one cycle lower Temp for anneal using pnNI and H'54 XhoRed. After PCR, cut EarI, Blunt, XhoI

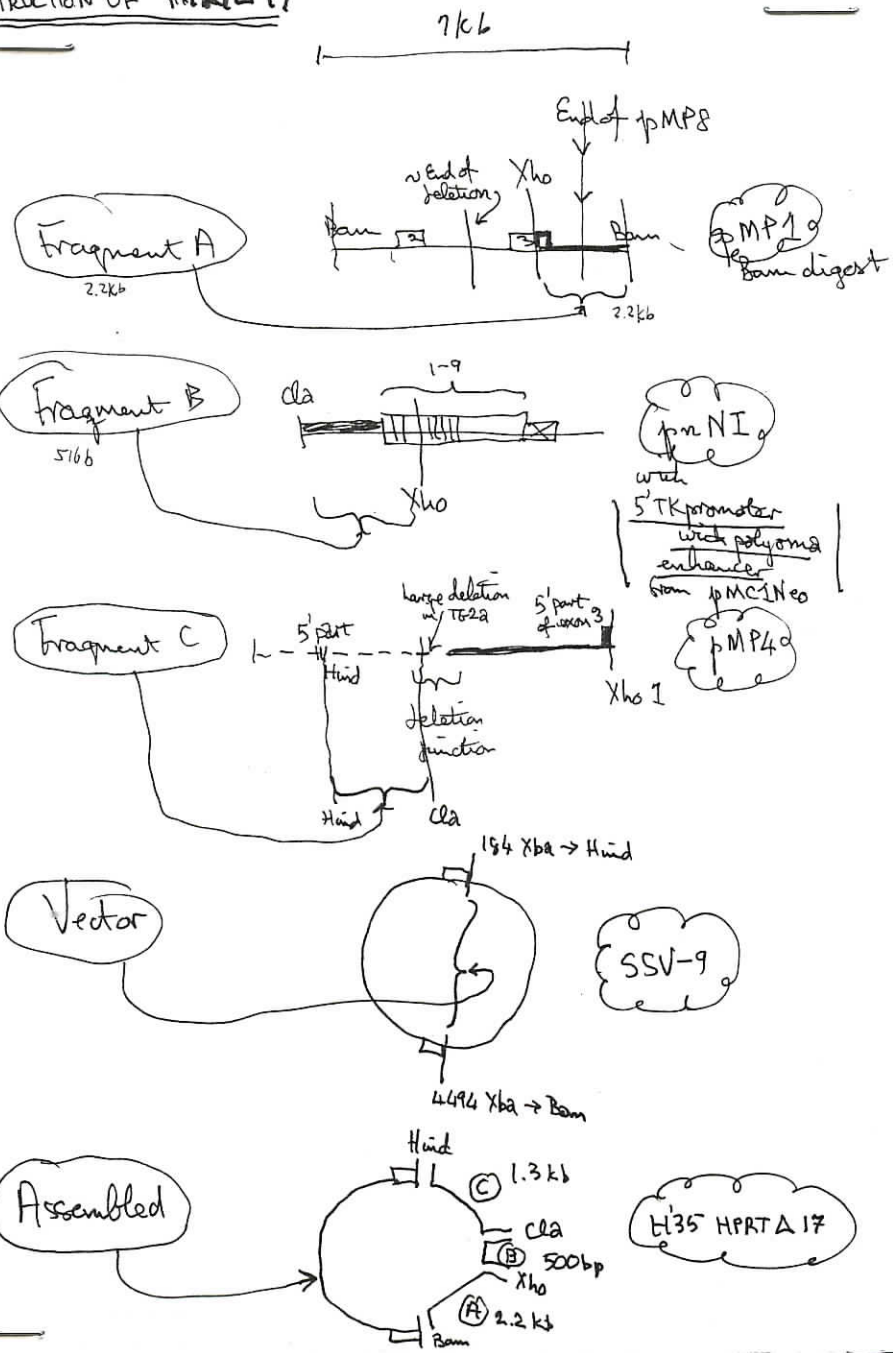


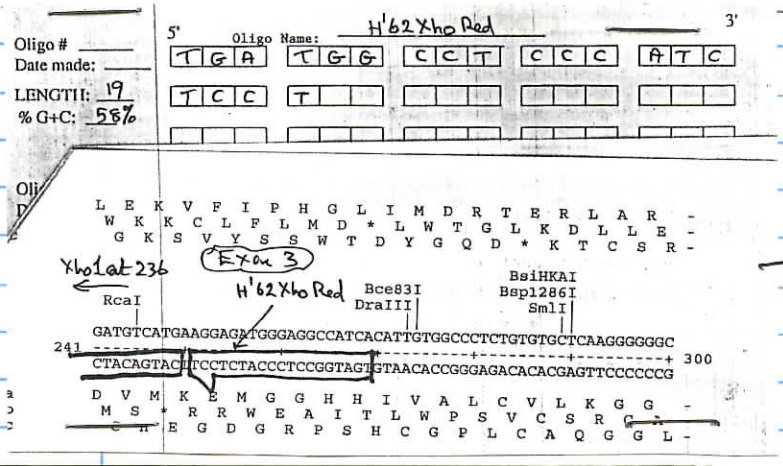
Safer is two primers

Secondary primer AACTCTTCGCGTCGTGATTAG
Tm = 60°

and first primer AACTCTTCGCGTCGTGATTAGGATGAT
T in human i.e. in pnNI
Priming T_m = 60°

CONSTRUCTION OF HPRTΔ17





into 500 µl ✓ 0.001M EDTA
1x CIAA ✓

H'62 Xho Red →

Reseal
shows
a poor

Sun - July 26th

H'53 | HPRT Δ9 (i.e. with only 2 Cla sites)

Data from H'35 | HPRT Δ17 tests

Seigo finds using 10 μg of H'35 HPRT Δ17 (and H'53 Δ9 each) cut w/ BamHI, no colonies with HM-1 ES cells. Positive control = for h.r. using pMP8 gave ~950 colonies. Checked the construction (see left) & can find no errors. Possible problems are that:-

a) the construct yields a recombinant that lacks any HPRT-enhancer (in intron 2)

b) ...that has a poor promoter (pMC1neo).

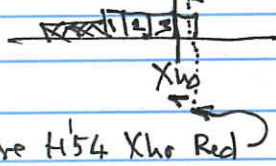
[Note that pvuII 2 gave 5,000 colonies - so promoter + enhancer work ok]

∴ check packaged form, to increase frequency and switch fragment (B) to PGK promoter.

Also re-design h.r. construct to include enhancer in intron 2 and includes natural HPRT promoter

Meanwhile do a little sequencing on Δ17 using

primer from exon 3. → H'62 Xho Red (see left)



- or could use H'54 Xho Red

Mon. July 27th

Sequencing Check on H'53 HPRT Δ9

To sequence as July 26th (#66)

Standard protocol θ 101/143

Result 7/29 See H66

Shows correctly is okay, but a poor sequence, so details are not good.

1.3 μg/μl

H'53 Δ9 DNA → 3μl + 16.5μl 0.001M EDTA

Water → 10μl ✓

H'62 Xho Red/100 → 5μl

1 μg/5 μl

H'63 Δ9 Dil'd

Std. prep'n. in 500μl 0.001M EDTA

5μl ✓

New primer for opposite strand to confirm 7/29 data

(#67) H63D9

- H'53 Δ9 DNA diluted 5μl
- Water 10μl
- H'63 MP8/5'Blue 5μl ✓ 1/100

COMMENTS:

Oligo #
Date made:
LENGTH: 21
% G+C: 43%

5' Oligo Name: H'63 MP8/5'BLUE 3'

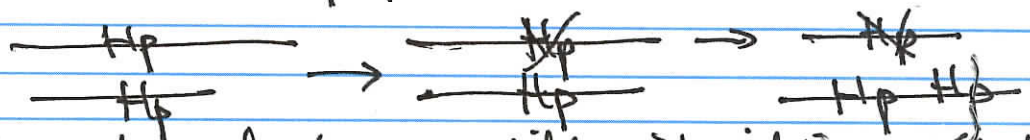
A	C	G		T	C	A		G	T	A		G	T	C		A	T	A				
G	G	A		A	C	T																

5μl → 495μl 0.001M EDTA → H'62 Xho Red/100

Wed. July 29th

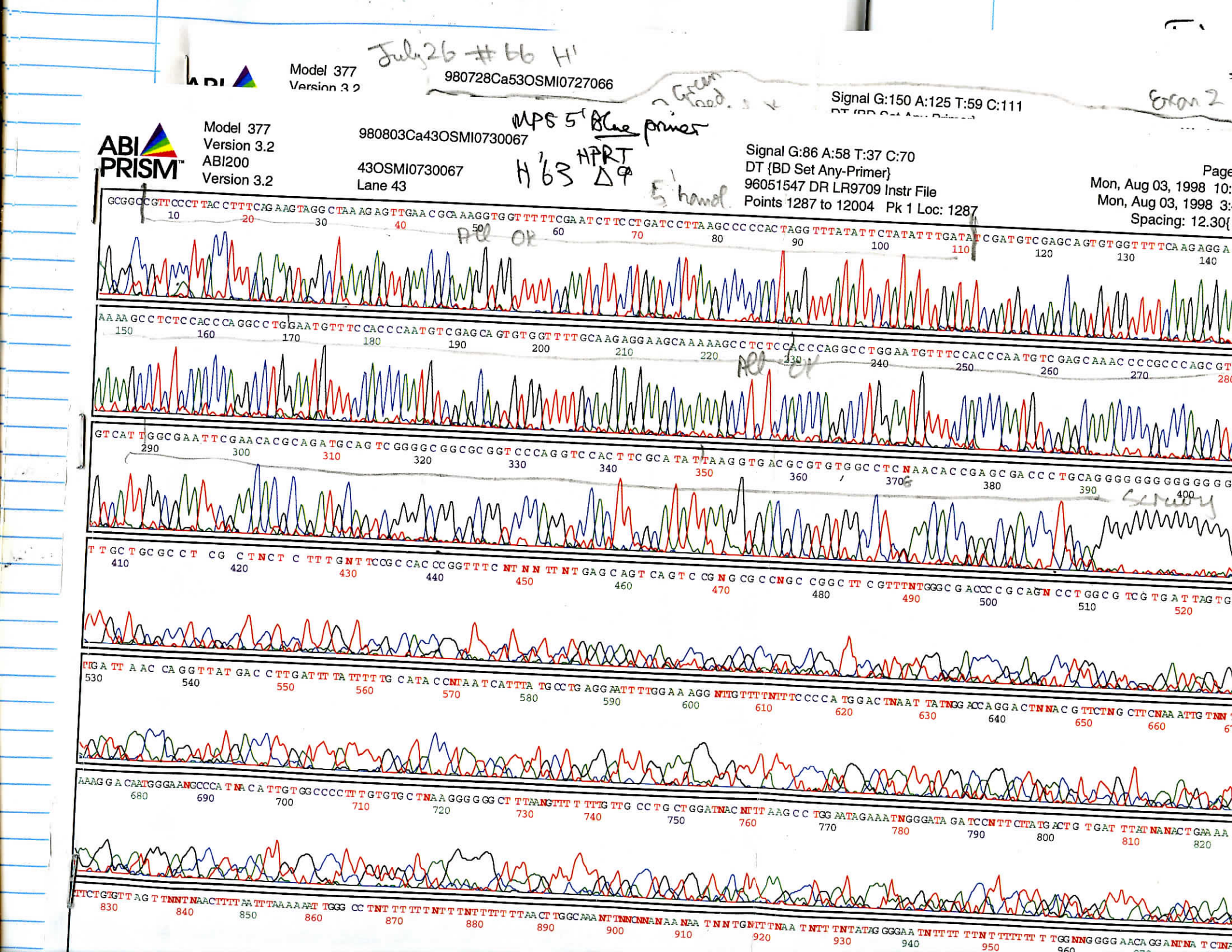
Haptoglobin knock-out

Review of Randy T's multiple features to obtain transmission of 129 genome (no agouti!) with Hp 1/0 ES cells suggested possible importance of Hp in Fe transport. Mediate revealed expression in uterus and in Sertoli cells (R.T.). Possible corrections are to make an Hptransgene in the Hp1/0 ES cell to allow initial germline transmission & then use of transmission ± of transgene for future breeding etc. An improved thought is to make the ES cell line Hp 2/0 i.e.



but need to check on possible imprinting complications.

Refs: Olson et al. J. Endocrinology 152, 69-80 (1997)
Specific expression of hp in implantation-stage rabbit uterus epithelium
O'Bryan et al. J. Andrology, 1997 18, 637.
Hp is a Sertoli cell product in the rat etc. etc.


July26 #66 H'
Model 377
Version 3.2
980728Ca53OSMI0727066
Signal G:150 A:125 T:59 C:111
Exon 2
MPS 5' Blue primer
ABI PRISM
Model 377
Version 3.2
ABI200
Version 3.2
980803Ca43OSMI0730067
43OSMI0730067
Lane 43
H'63 HPRT Δ9
5' homol
Signal G:86 A:58 T:37 C:70
DT {BD Set Any-Primer}
96051547 DR LR9709 Instr File
Points 1287 to 12004 Pk 1 Loc: 1287
Page
Mon, Aug 03, 1998 10:
Mon, Aug 03, 1998 3:
Spacing: 12.30
GCGGCCGTTCCCTTACCTTTCAGAAGTAGGCTAAAGAGTTGAACGCAAAGGTGGTTTTTCGAATCTTCCTGATCCTTAAGCCCCCACTAGGTTTATATTCTATATTTGATATCGATGTCGAGCAGTGTGGTTTTCAAGAGGA
AAAAGCCTCTCCACCCAGGCCTGGAATGTTTCCACCCAATGTCGAGCAGTGTGGTTTTGCAAGAGGAAGCAAAAAGCCTCTCCACCCAGGCCTGGAATGTTTCCACCCAATGTCGAGCAAACCCCGCCCAGCGT
GTCATTGGCGAATTCGAACACGCAGATGCAGTCGGGGCGGCGCGGTCCCAGGTCCACTTCGCATATTAAGGTGACGCGTGTGGCCTCNAACACCGAGCGACCCTGCAGGGGGGGGGGGGGG
TTGCTGCGCCT CG CTNCT C TTTGNTTCCGC CAC CCGGTTTC NTNN TTNTGAGCAGT CAGTCCGNGCGCCNGC CGGCTT CGTTTNTGGGC GACCCCGCAGN CCTGGCG TCGTGATTAGTG
TTGATT AAC CAGGTTAT GAC CTTGATTTATTTTG CATACCNTAATCATTTA TGCCTGAGGAATTTTGGAAAGG NTTGTTTTNTTTCCCCA TGGACTNAAT TATNGG ACCAGGACTNNAC GTTCTNG CTTCNAA ATTG TNN
AAAGGACAATGGGAANGCCCA TNACATTGTGGCCCCTTTGTGTGCTNAAGGGGGGCT TTAANGTTT T TTTGTTG CCTGCTGGATNAC NTTTAAGCCTGG AATAGAAATNGGGATA GATCCNTTCTTATG ACTG TGAT TTATNANACTGAA AA
TTCTGTGTTAGTTNNTNAACTTTTAATTTAAAAAT TGGG CC TNT TT TTTNTTTNTTTTTTAACTTGGCAAANTTNNCNNANAANAA TNNTGNTTTNAA TNTT TNTATAG GGGAA TNTTTT TTNTTTTTTT TGGNNGGGAACAGGANTNA TCNA

Fri. July 31st

HPRT Δ9 sequence → DWM110

Correction! Re-read sequence shows it is okay.

The rather poor sequence from July 26 #66 shows general assembly is correct, but a real possibility of a frame shift mutation in Exon 1 is apparent. Re-sequence in opposite direction using a different primer H'63 M#8/5' Blue || H'63 (#67) does this but blocks at Xho 1 Seigo's new Cla I PB-K promoter 1213 polyG from David Melton's pDWM1 (H'8) will correct this error.

Meanwhile planning to make a new h.t. construct with Cla — Xho replaced by mouse HPRT promoter, Exons 1 & 2 truncated intron 2 Exon 3 Xho. Data available from DWM110 map & construction is somewhat difficult to interpret ∴ sequence out in both directions from Exon 1 using following primers:—

Exon 1 (data from Gen Bank

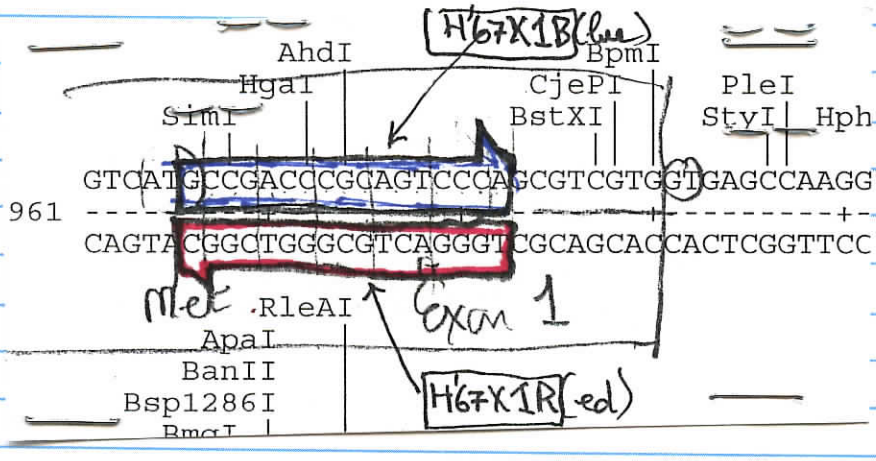
... Met Pro .... (GT) — MUS HPRT PROMOTER)

... intron

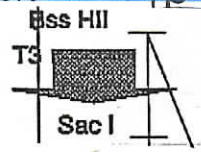
Oligo # 9735 5' Oligo Name: H'67X1Blue 3'
Date made:
LENGTH: 19
% G+C: 76%
G C C | G A C | C C G | C A G | T C C
C A

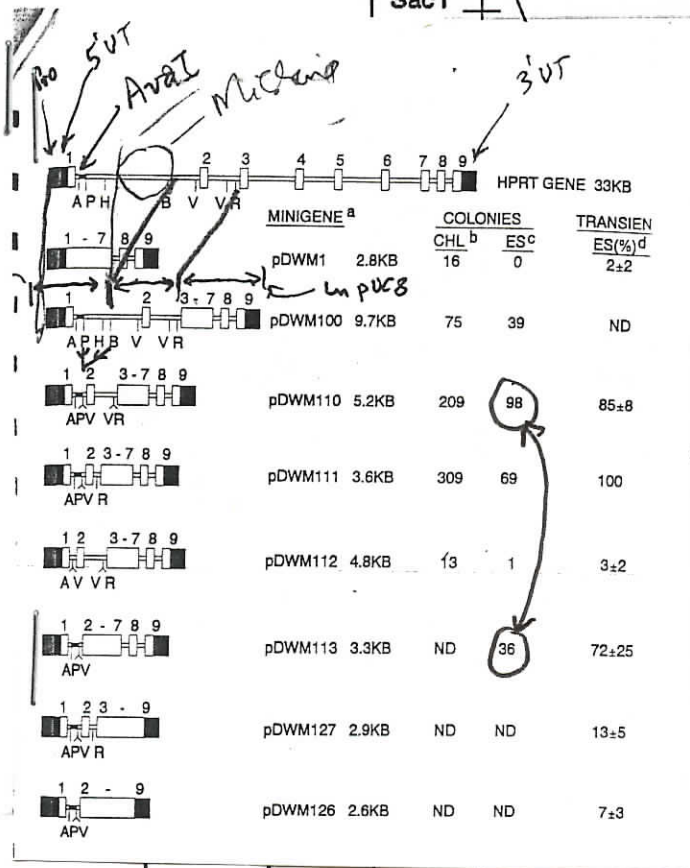
Oligo # 9736 5' Oligo Name: H'67X1Red 3'
Date made:
LENGTH: 17
% G+C: 76%
T G G | G A C | T G C | G G G | T C G
G C

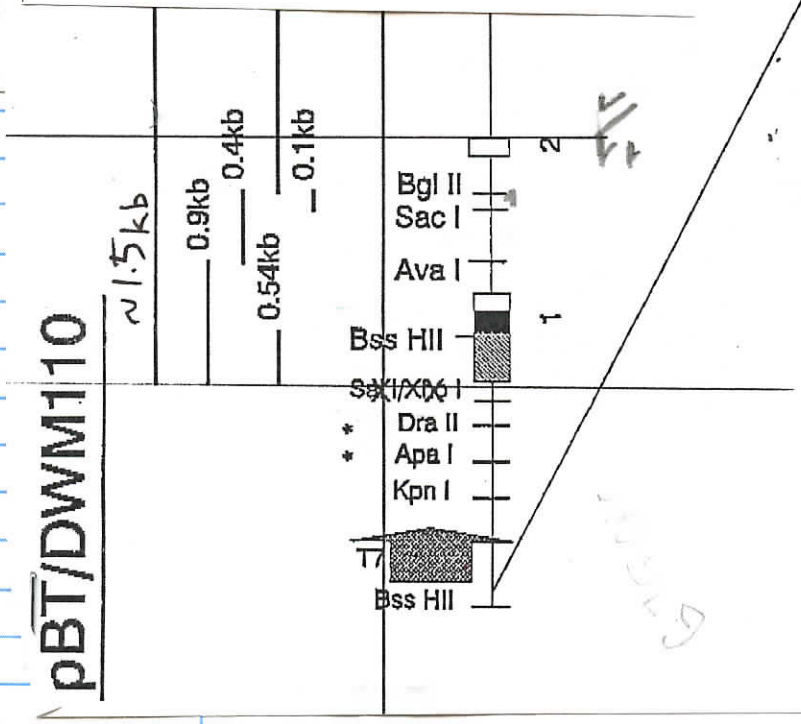
Sequence Rx #71
H'67 HP9
H'63 Δ9 Dil'd 5 µl ✓
Water 10 µl ✓
H'75 HPRTΔ Blue 5 µl ✓
1/100

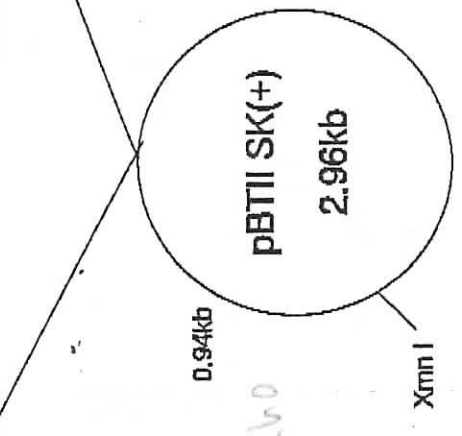


Primers etc. for H'69









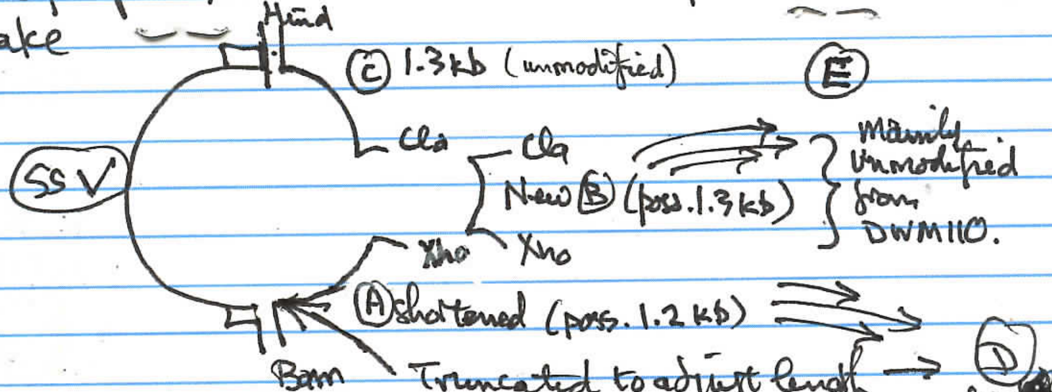
HPRT promoter
HPRT untranslated
HPRT coding
* unmapped cut/ no information within insert

DWM110. S89

Fri. July 31st — Dad's birthday

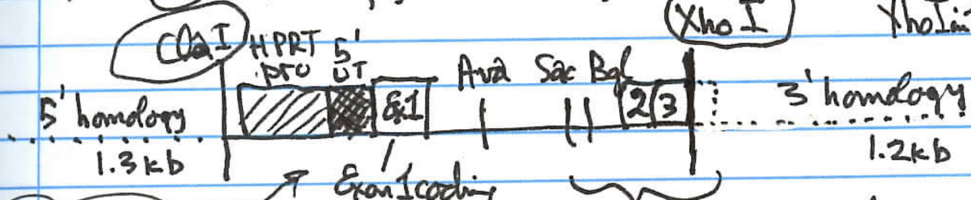
Mus HPRT pro Intra H.R. construct

Using pBT/DWM110 as a basis, I should be able to make



New (B) will be, if sites allow, :–

XhoI in pMP8 to Sal → Bam



This sh'd be about 36/48 as good as DWM110 & many times better than no intron

Make this piece with

Sac or Bgl → ← Xho (40bp 1° primer, 20bp 2° primer) using DWM110 as template.

H'54 Xho Red or new

Mon. Aug 3rd

Sequence data needed for construction – using H'67 X(Exon1) Blue & Red primers :–

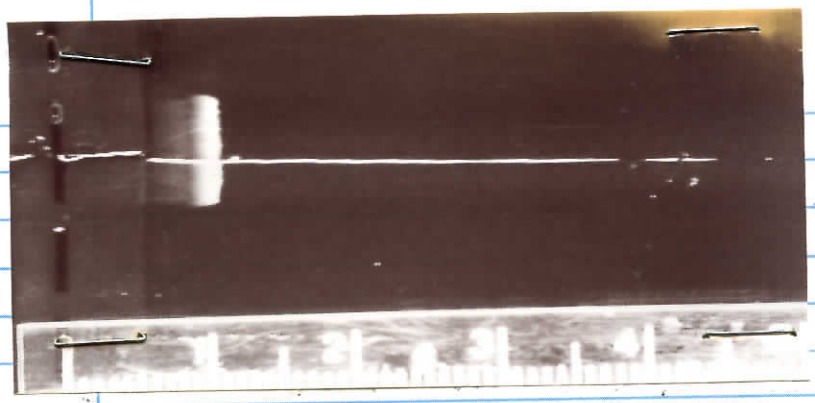
H'69 #68	H'69 #69
1μl ✓	1μl ✓
14μl ✓	14μl ✓
H'67X1 Blue/100 5μl ✓	H'67X1 Red/100 5μl ✓

Sent Aug 3rd

"5' DWN110 F66" presumed DWM110 minis from Seigo H'69 — To miscellaneous

Water

(See page



① U.V. et. Br. stain after cut was also good.

To Miscellaneous

H'73 R1
pSKB1 35
1.4µg/µl
modified by Scott

(1)

On 5:55 pm
Off 11 am 0.8% HX4
1V/cm

Dye
Gap
8 slots
H'73
SKB1
Sal
Lig
Water
Dye

Tues. Aug. 4th

pMP8 Sal to Bam

H'73

Take 1.4 µl (2 µg) of pSKB1 (pMP8SKB modified by Scott)
1.4 µg/µl

87 µl ddw
10 µl 10x R1 salts
1 µl Sal I Lot 66 (10u/µl) 0.2 µg in 10 µl

H'73 SKB1 Sal

37° 5:18 to 8:10

10 + 8 µl → PRE H'73

10 + 8 µl → H'73 Post Sal ← but left at rt overnite!

Whole (80 µl) + 10 µl 10x p95 + 10 µl H'68 Sal to Bam oligo
+ 5 µl T4 ligase (dilute) Lot 67 → H'73 SKB1/LIG^N

4° 8:17pm to Tues 4:30 pm Wed

Gel purify if yield on transformation is low.
– or as back up.

5 µl to 15 µl 1x PBS
~60° for 5 min.
Ice

Transformation

H'35 DH5α 80 µl
Heat 1 SKB1 Lig^n 5 µl

0° 4:48 pm to 5:25   3pm to 3:30pm Th.
H'73² plated at 37° 3:50pm

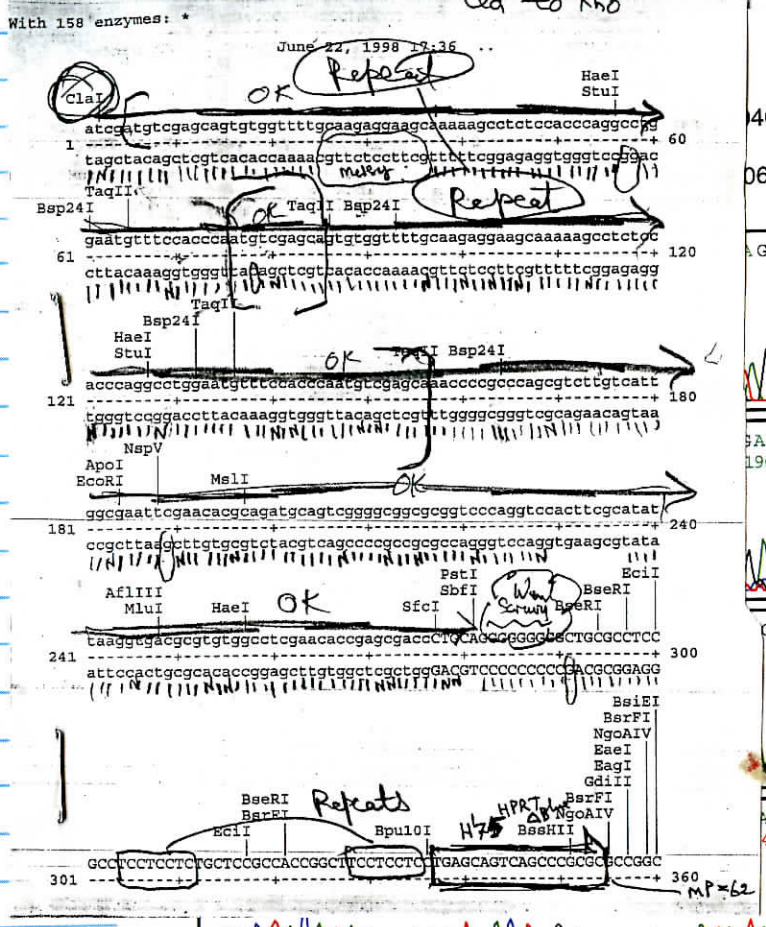
42°C 90 sec.

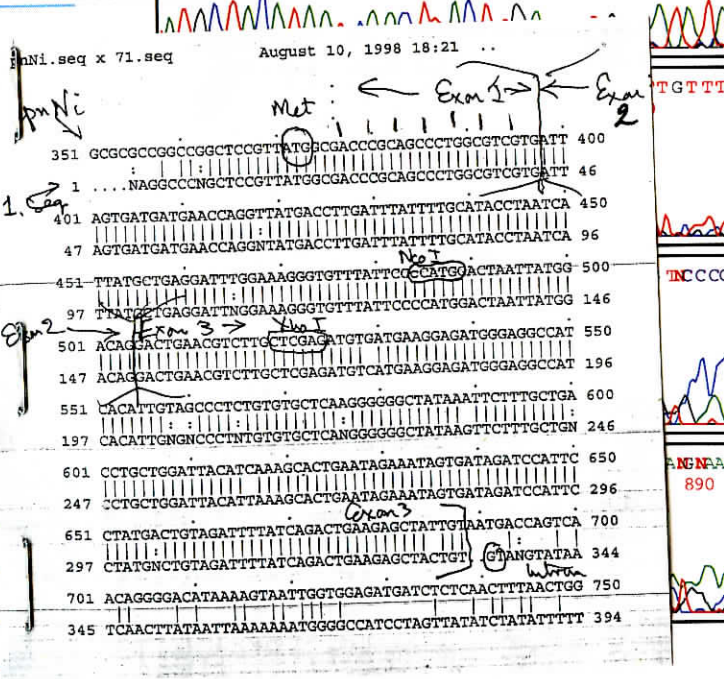
(720) (4)

Plate 1x AMP N2Y Rest, 20, 5, 20/16, 5/16 37° 5:35 pm

→ Processed to H'73 SKB1 Bam Conc in 50 µl 0.001M EDTA
Transform with 5 µl

H'73 Minis #1 – 12 37° 3:30pm ← Thurs. 37° [illegible] to 9 am Fri.
Processed to 50 µl (Contd. on page H'79).





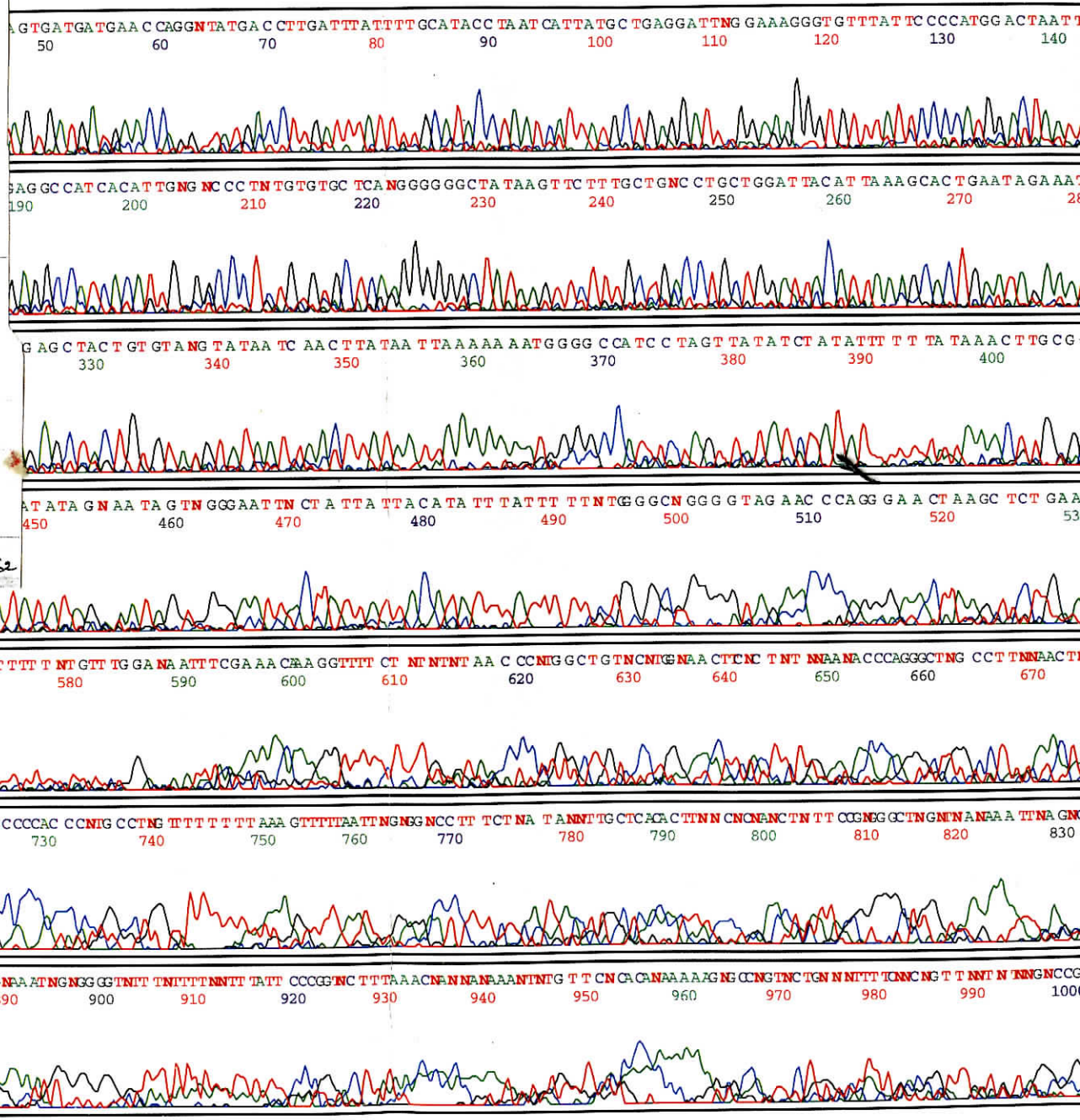
H75 DNM 2C6
Blue

4OSMI0806071

06071

Signal G:43 A:40 T:26 C:33
DT {BD Set Any-Primer}
96051547 DR LR9709 Instr File
Points 1330 to 12006 Pk 1 Loc: 1330

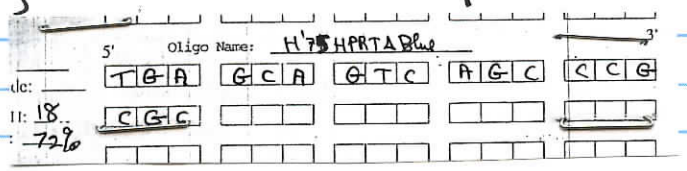
Sun, Aug 09, 19
Fri, Aug 07, 19
Spacing: 11.9



Wed. Aug 5th

Final(?) check on HPRT Δ9

Still not happy about coding region of Δ9 because second sequence quit at GGG..., and first sequence was poor & incomplete in coding. * sequence further in the Blue direction using H'75 HPRT Δ Blue primer



H'67 HP9 seq. (71.seq) with H'75 HPRT Δ Blue checks out fine.

H'75 DWM110 Red 250

ABI PRISM™

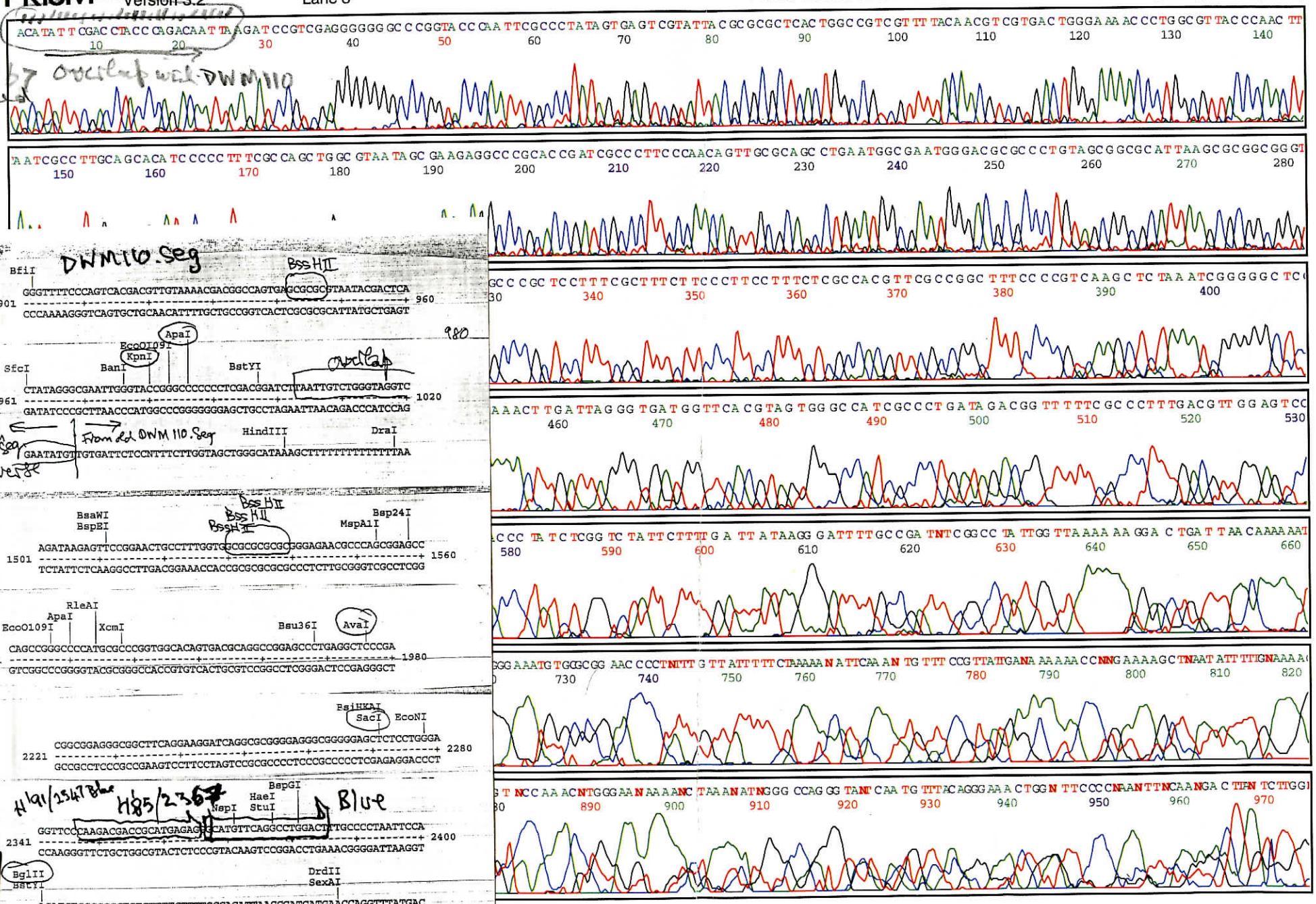
Model 377
Version 3.2
ABI200
Version 3.2

980807Ca030SMI0806070

030SMI0806070
Lane 3

Signal G:104 A:85 T:54 C:95
DT {BD Set Any-Primer}
96051547 DR LR9709 Instr File
Points 1336 to 12006 Pk 1 Loc: 1336

Sat, Aug 08, 1998 0:07
Fri, Aug 07, 1998 17:23
Spacing: 11.99{11.99}



Wed. Aug 5th

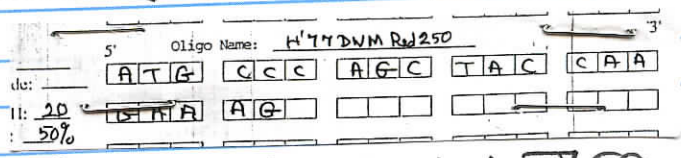
DWM 110 Sequencing

>750 bp from exon 1 in red direction agrees with mus HPRT promoter sequence, but it breaks at ~~~~ bp200 in MUSHPRTPR. So make new red primer to continue, starting at :-

H'77 DWM Red250

241 ATGCCCAGCTACAAAGAA 275 MUSHPRTPR

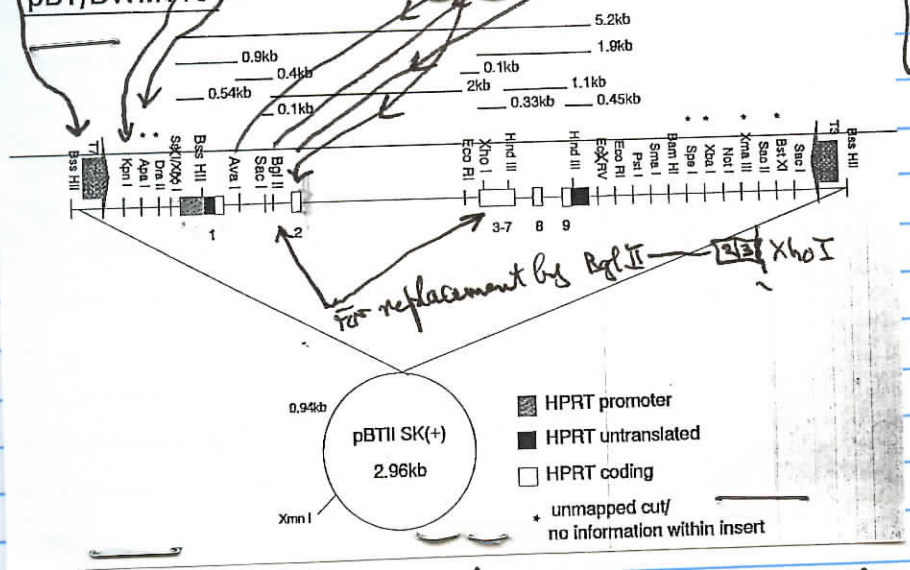
250



Meanwhile the blue sequence from exon 1 picks up at Exon 1 (GT)... → & is available as needed. The AvaI and SacI and BglII sites are in sequence & whole of exon 2. Sequence H'77 #70 ✓ completed the story well

BssHII, KpnI, ApaI

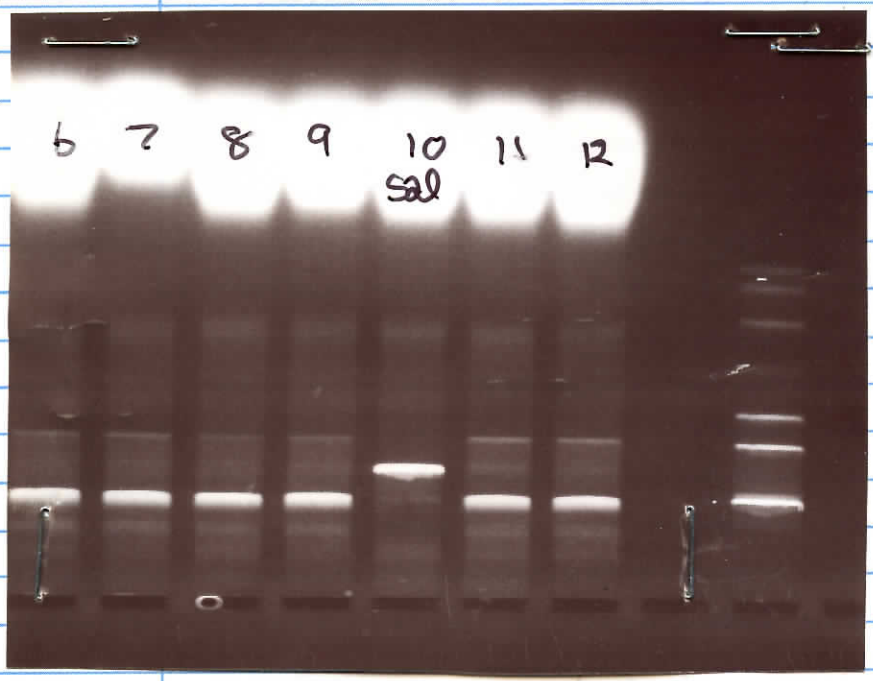
pBT/DWM110

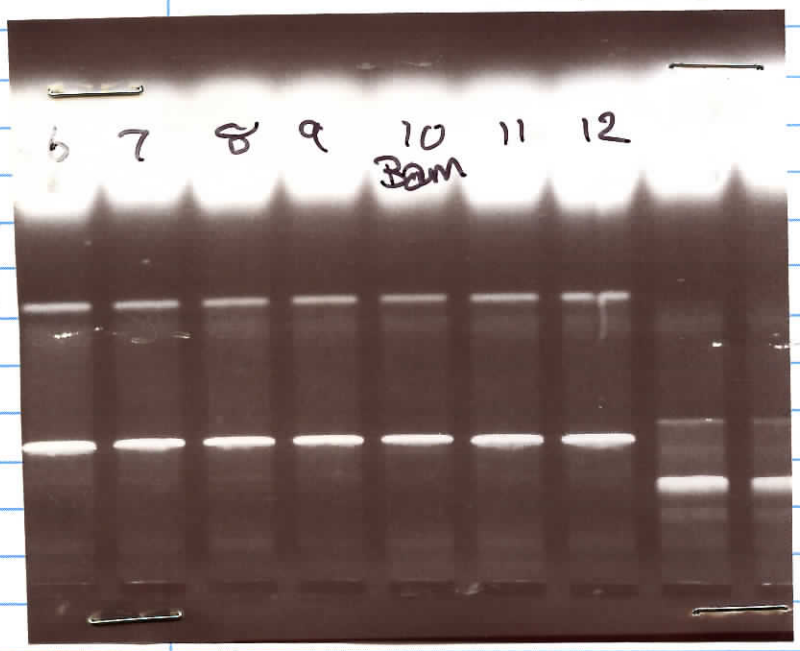


H'77 #70 sequence

H'69 DWM 110 mini 5	1 µl ✓
Water	14 µl ✓
H'77 DWM Red 250 primer x 1/100	5 µl ✓

Test possibility of changing KpnI to ClaI & using PCR to pull out BglII to Exon 3 (added on).





① Conclusion: All except #10 lost the Sal sites - as desired - but don't appear to have gained a Bam. One interpretation is that there is a Bam site close to the Sal site & that appear to be same as two. But SKB1 Alternately - all have gained the Bam, but #10 did not lose both Sal sites.

H'79.
Pooled Minis
1-8 11 & 12

H79 Mini 9 used

On 12:18pm Off 1:18pm 0.8to HyLeMAX

Fri. Aug 4th

H'73 minis SKB Sal → Bam

Take ✓26µl 10×R1 salts 26µl ✓
✓232µl ddw 230µl ✓
2µl Bam HI lot 77 (20u/µl)
Sal I lot 66 (9u/µl) 4µl ✓

20µl Each + 1µl H'73 minis #1–12 20µl

Bam digest H'73 minis

Sal digest H'79 Bam

H'79 Sal

37° 10:43am to ~11:50am Back back until 2:40pm

Sat. Aug 5th

Conclusion: Proceed to test ability to excise Xho Bam of predicted size (1.2kb) with a single Xba I site that cuts it into (633bp + 560bp)

Take 30 µl 10×R1 salts
µl ddw
270
2 µl Bam HI lot 77 (20u/µl)
1.3 µl Xba I Lot 30 (20u/µl)
0.7 µl Xho I lot 30 (20u/µl)

Back
30µl 10×R1
240µl ddw
3µl Bam HI
3µl Xho I
30µl mini #9
37°
11:23am to 2:40pm

3×20 70µl 70µl 70µl 3×20 3×20

10 SKB

Bam Xba+Bam Xba+Bam+Xho

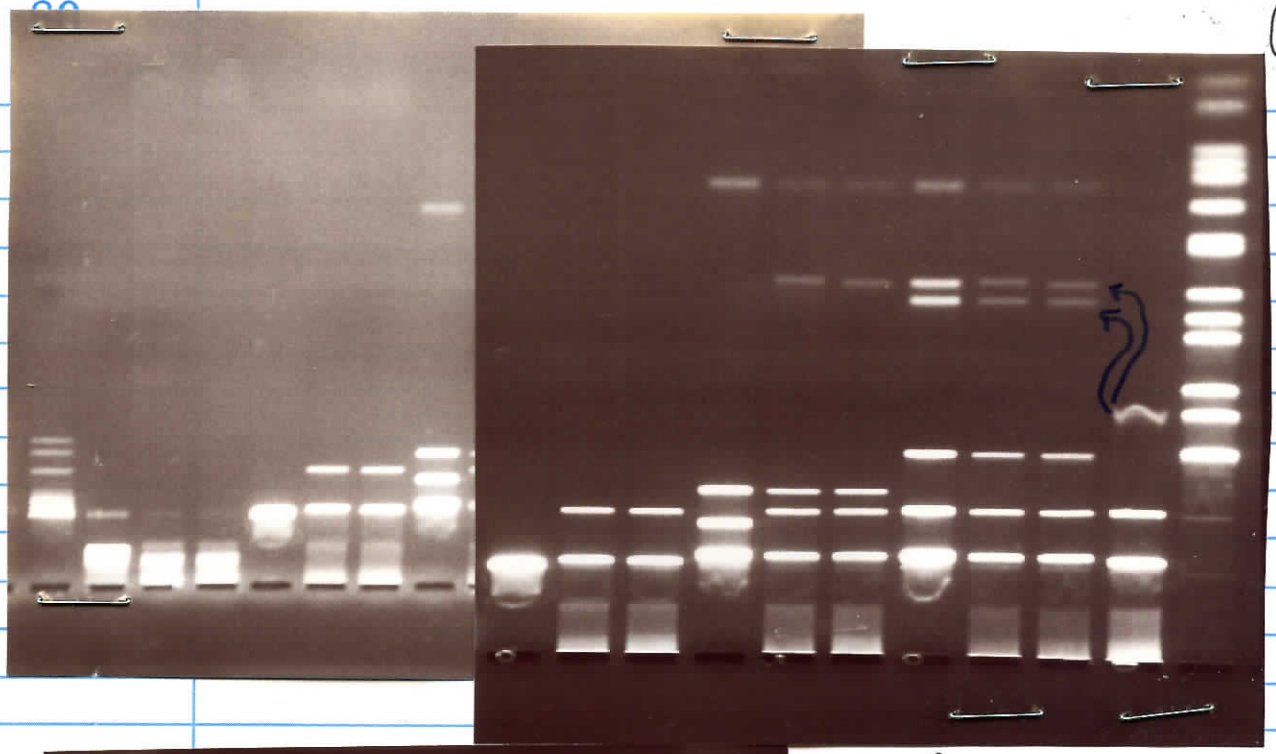
1µl mini 9
1µl mini 10
2µl pMP8 SKB H73 diluted 1/4
10µl + 30µl
1:4

9 10 9 10 9 10

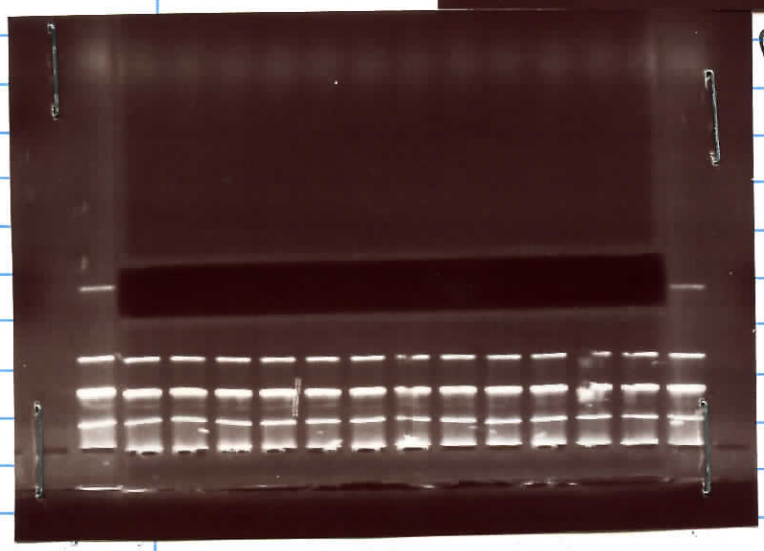
SKB SKB SKB SKB

37° 11:23am — 12:40pm

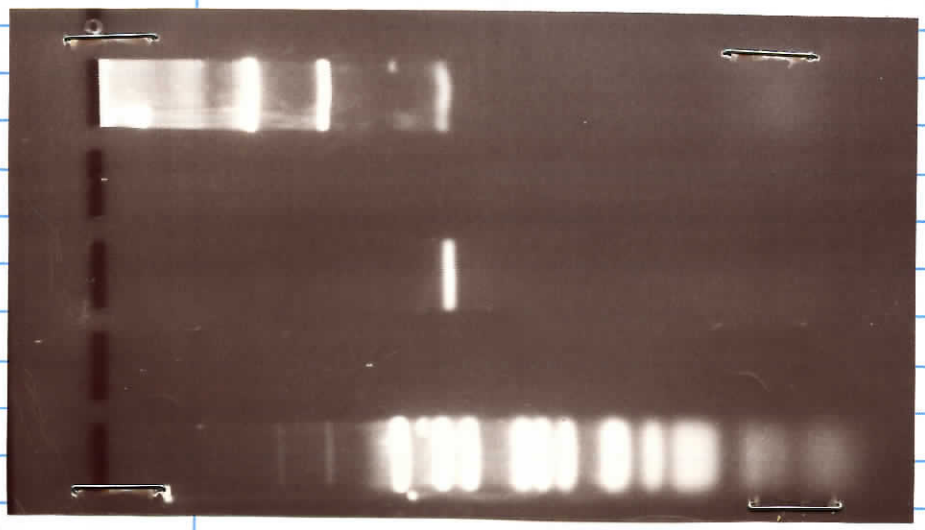
H'79 B/X



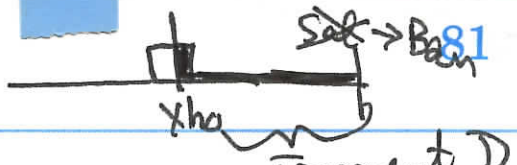
(1)



(2) Rather unbrave cut!



(3) Nice product!



① On 1:02 pm Off 1:32 pm 2% HX1 2 µl RNAse per 10ml 10V/cm

Sat. Aug 8th

Dye
Water
AG MM

H'73 minis SKB Sal → Bam

SKB Mini 9	Pre
Mini 10	Digests (p. 79) rem.
SKB Mini 9	Bam
10	
SKB 9	Bam + Xba
10	
SKB 9	Bam + Xba + Xho
10	

Conclusion SKB has a single Bam site. Bam + Xba on 9 & 10 as 560 bp as expected, and slightly longer in 10). Bam + Xba + Xho gives also the 633 bp band (Xho replaces Bam in SKB) in all 3. Bam + Xho gives the expected/desired 1.2 kb band although ep. is odd.

Bulk 9 Bam + Xho
ACR MM
W
Dye

② On 10.45 am Off 11:45 am 5V/cm

Dye
Water
1.5% HX1

Wells with Bulk H'79 9 B/X

Recovered as

H'81	in 75 µl
X to B	0.001 M
FRAG D	EDTA

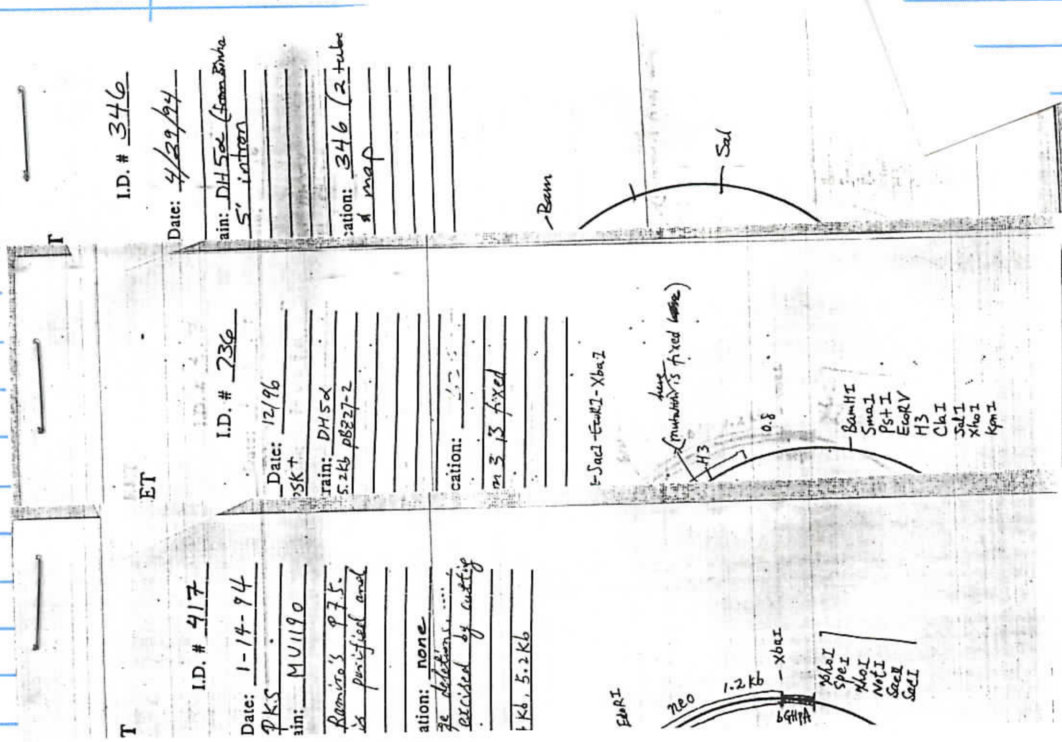
1.2 kb

Water
Dye
G

③ On 1:43 pm 1.5% max 9 HX1 RNAse

Dye
W

Bulk H'79 9 B/X
H'81 X to B Frag. D
W
ACR MM
W
Dye



07/28/98 11:18 919 966 6718

THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL

The School of Medicine
Department of Pathology
Telephone (919) 966-6876
FAX (919) 966-6718

O. SMITHIES
The University of North Carolina at Chapel Hill
CB# 7525, Brinkhous-Bullitt Building
Chapel Hill, N.C. 27599-7525

July 28th, 1998.

Dear Alan,

We seem to be reaching the upper limit of the size of duplications that can be made with the gap-repair method (PNAS 91, 3612, 1994) — somewhere around 100 kbp. We thought we might try your Cre-lox system as published in Nature 1995, 378, 720. [Since we are working with a single gene we can construct the B> B> type of targeted cell with a 50% yield]. It would be very helpful to us if you could provide us with plasmids & maps containing the two necessary cassettes (presumably 3 and 5) Figure 1). We would not transfer them to anyone else & would not be using them in conjunction with any commercial activities. In the (unlikely) event that any mice made with their use had any commercial value we would get in touch with you to reach some mutually satisfactory agreement.

Keep up the good work,

All the best,

Oliver.

Hejv — please send the plasmids you sent out recently for Chr. engineering.

All —

Fri. Aug 7th

Received Payments from Allen Bradley to make CRF/TOX duplications.

H'85

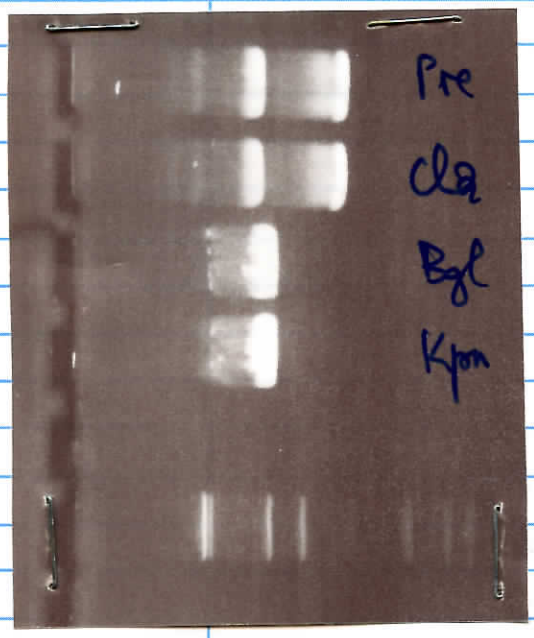
19836 5' Oligo Name: Kpn to Cla (noGATC) 3'
Oligo #: ____
Date made: ____
LENGTH: 14
% G+C: 43%

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

19837 5' Oligo Name: H'85 /230[illegible] Blue 3'
Oligo #: ____
Date made: ____
LENGTH: 19
% G+C: 58%

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

3'



① Conclusion Cla does not cut; Kpn and Bgl II cut simply — all as expected. Proceed.

① 1:45
Dig
W
Pre
Cla
Bgl
Kpn
W
Ag M
W
Dig

① 1:45 pm
On 12:45 pm Off ~~.8%~~ 0.8% g H x 4 / 10 ml RNAse
2 μl MAX

Dye
W
Pre
Cla
Bgl
Kpn
W
Ag MM
W
Dye

all

Tues ~~Aug~~ 11th

DWM110 digests

Expecting to modify KpnI site to ClaI, & later excise PCR product with Bgl 2.

∴ take ✓ 16 μl 10 x RI salts
✓ 136 μl ddw
✓ 8 μl H'69 DWM110

H'85 Pre	H'85 KpnI	H'85 ClaI	H'85 Bgl 2
18 μl	~~[illegible]~~ Rest	18 μl	18 μl
	+ 2.5 μl ✓	+ 1 μl ✓	+ ½ μl ✓
	KpnI Lot 48	ClaI Lot 33	Bgl 2 Lot 35
	10 u/μl	5 u/μl	10 u/μl

H'85 DWM110 Kpn

37° 11:38 am – 1:35 pm

Amplification & extension of Bgl2

201 bp → [2|3] Xho

41 nt
2527 + 41
− 2367
201 bp

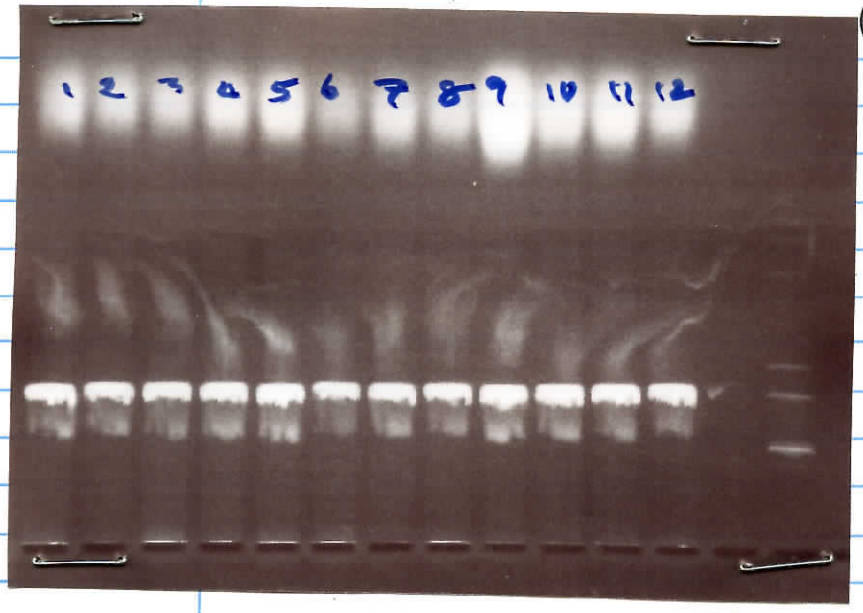
Primer from 3' of Bgl2 chosen from H'~~77~~ Seq #68

GCATGTTCAGGCCTGGACT

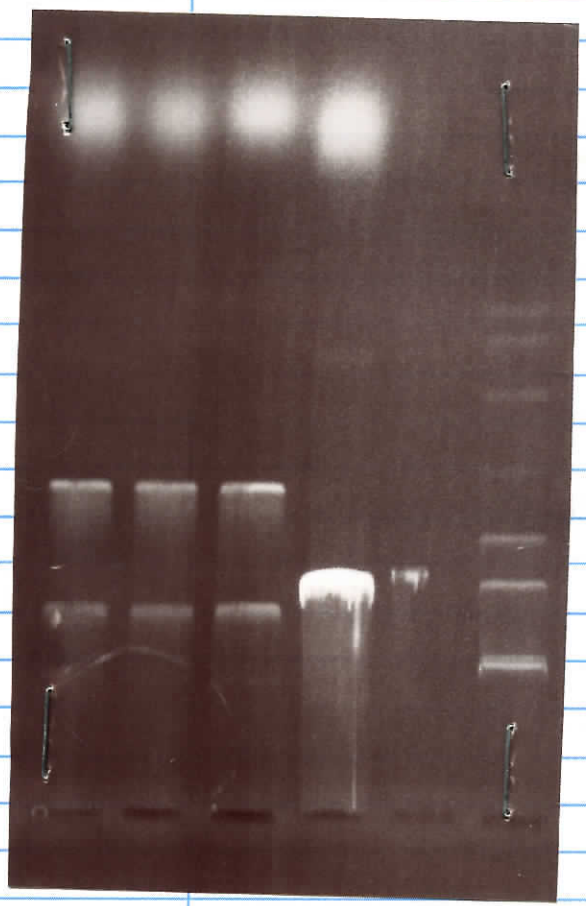
11 x 4 = 44
8 x 2 = 16
60

H'85, 23~~67~~ Blue

(see 23~~67~~ DWM110.seq)



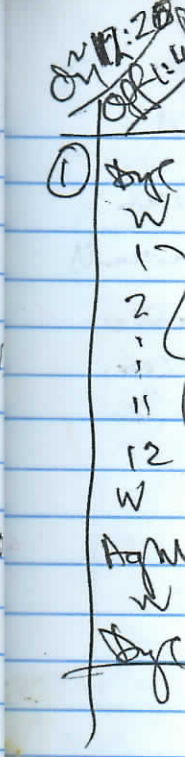
① Conclusion: Minis were OK — another 100% ! Part a possible problem is 9 but not Lot DWM 110 with Cla in correct buffer Kim pointed out that Cla I cuts in all the usual buffers. But see H'8 9 — no cut of 11 in buffer 4

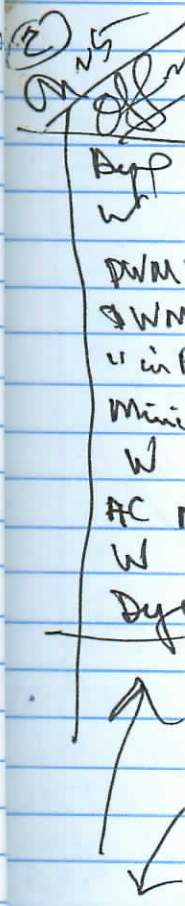


② DWM 110 doesn't cut with Cla in either. Mini 4 cuts with Cla in R 1 So all are OK!

Pooled minis
H'87 1-3 5-12

H'87 #4 used





DWM 110
Control, Cla
Cla in buff
Mini 4 in R
37° at 2:

Kpn → Cla

Wed. Aug 12th

Kpn I to Cla I in DWM110

Take ✓ 2 µl p95 10× buffer
✓ 15 µl ddw
2 µl H'85 DWM110 Kpn —— H'87 LIGN
✓ 1 µl H'85 Kpn to Cla oligo
✓ 1 µl T4 ligase dilute lot 67

15° 9:50 am to 2 pm, RT 2 pm to 3:20 pm, 37° 3:20 pm to 4:35 pm

5 µl + 15 µl 1×PBS ~60° for 2-3 min – ice

H'35 DH 5α 80 µl ✓
Heated ligⁿ H'85 5 µl ✓
0° 4:50 pm to 6:10 pm ✓

42° 90 sec (>30) (6)

Plated Rest, 20, 5, 20/16, 5/16 37° 6.20 pm
× Amp NZY

H'87 Mini #1–12 to 37° 3:35 pm
1–2 × Amp NZY

Processed as usual but the ppt. was very thin + widespread!

Take ✓ 26 µl 10× NEBuffer 4
✓ 26 µl 10× Alb
✓ 195 µl ddw
✓ 13 µl Cla I lot 33 (50/µl)

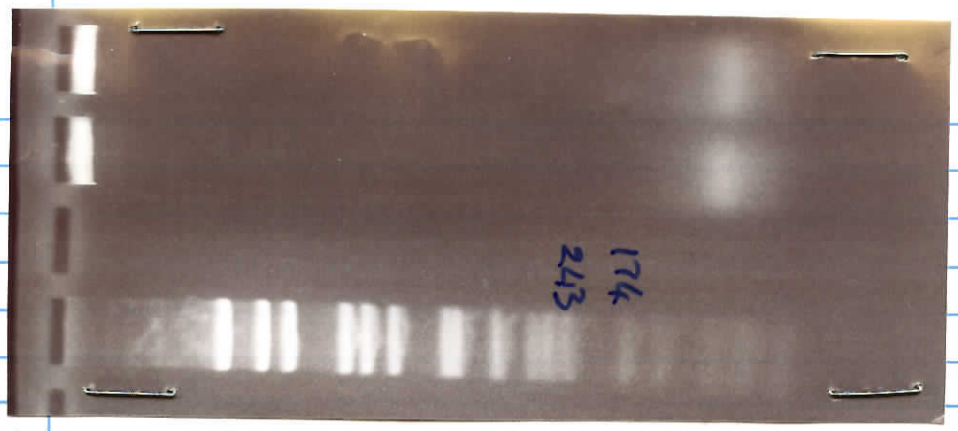
20 µl + 1 µl minis H'87 1 – 12 37° 11.08 am
12:08 pm

Check on Cla I

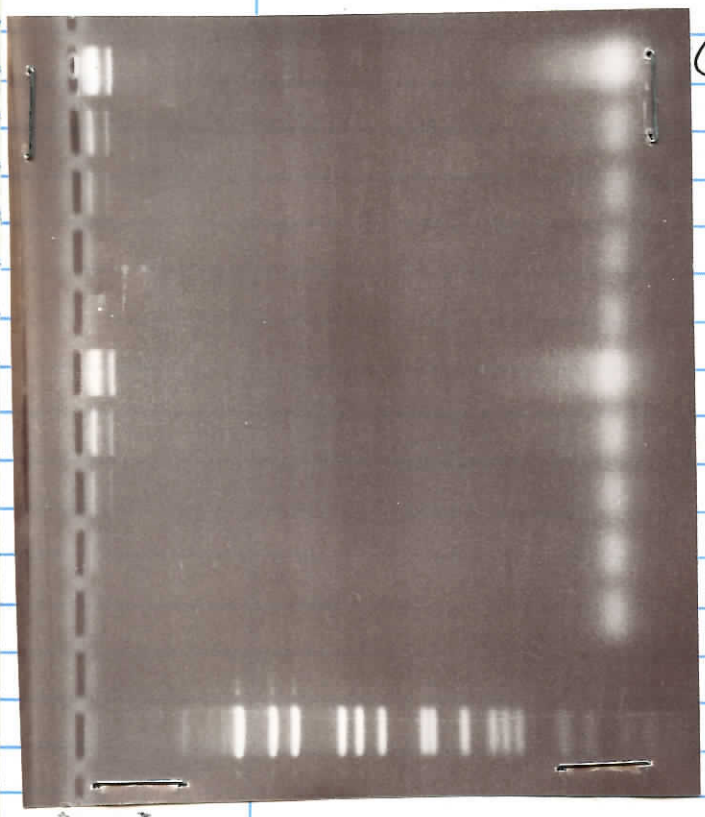
Take 2 µl 10× R I buffer / 2 µl Buffer 4 / 2 µl 10× Alb / 14 µl ddw / 1 µl H'87 mini 4 / 1 µl H' 69 DWM / 2 µl Cla I / 4 R.I.

(Left margin:)
0:12:20 pm 1:45 pm 0.8% HH4 MAX
① Dye W
1, 2, 11, 12 H'87 Cla minis
W Ag NMA W Dye
② 5 ... Buf W
DWM110 Cont.
DWM110 Cla in 4
" in R I
Mini 4 in R I
W
AC MN
W
Dye

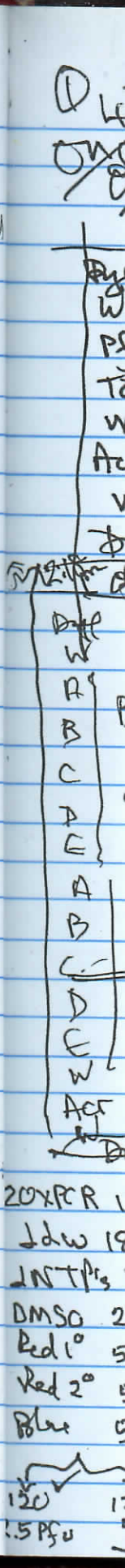
DWM110 H'69 Control, Cla in R I, Cla in buffer 4, Mini 4 in R I. 37° at 2:45 pm



① No product. Repeat with ↓ ng amount of template & single rxns. Ask Geiga to purify.



② Still no product. Remake red primer as 20 mer & add in old 1° & 2° as nested.



① 4:15 / 4:45 ~~Off~~ 2% H×1 MAX ~~On~~ 10V

Wed. Aug. 12th

Multiple Primer conversion of Bgl2 — [2], to Bgl2 — [2|3] Xho

(n = 10)

Water ✓	H'89	20× PCR	125 µl
Pfu Blue ✓		ddw	184 µl
Taq Black ✓	H'89	dNTP's	1 µl
W ✓	H'89	DMSO	25 µl
Act. MM ✓		H'68 Red 1° primer	5 µl
W ✓		H'68 Red 2° primer	5 µl
Dye ✓	H'69	[illegible] DWM110 mini	2.5 µl (~1 µg)
2% H×1 10V/cm ✓		H'85 / 2 357 Blue primer	5 µl

← Unfortunately left at room temp. for ~10 days.

[illegible]

120 µl ✓ → 2.5 µl H'89 Pfu polymerase, 4 Blue tubes ✓ (Stratagene lot 42 / 2.5U/µl)

120 µl ✓ → 5 µl Std. Taq polymerase, 4 Black tubes ✓

Blue Pfu: A, B, C, D, E

Black Taq: A, B, C, D, E

≥90° ≥1 min
35 cycles 93° 1 min
40° 7 sec
Nearer 59° → 60° 10 min

~11:50 am Start

After putting this Bgl 2 — [2|3] Xho1 fragment into DWM110 we will have a new mini gene that is ~3.3 kb long

Test it by e.p. in ES.

Thurs Aug. 13th

Same set up as 12th but with [illegible] varying template from (=1×, 1/3, 1/9, 1/27, 1/81) 1 µl of DWM110 H'69 1/4, 1/12, 1/36, 1/108, 1/324

2.5 to 1 µl 1/4, 1/12, 1/36, 1/108, 1/324 (Same as 12th)

A B C D E

60-61° extension

Started ~10:15 am

20×PCR	125 µl ✓
ddw	184 µl ✓
dNTP's	1 µl ✓
DMSO	25 µl ✓
Red 1°	5 µl ✓
Red 2°	5 µl ✓
Blue	5 µl ✓
120 / 2.5 Pfu	120 / 5 Taq

90

(1) Damn it! Still no product!! Review the primers

(1) 3:55 … 4:25 … 29 HX … Marx

Primer H '69 Sebaked Exon 2

ABI PRISM

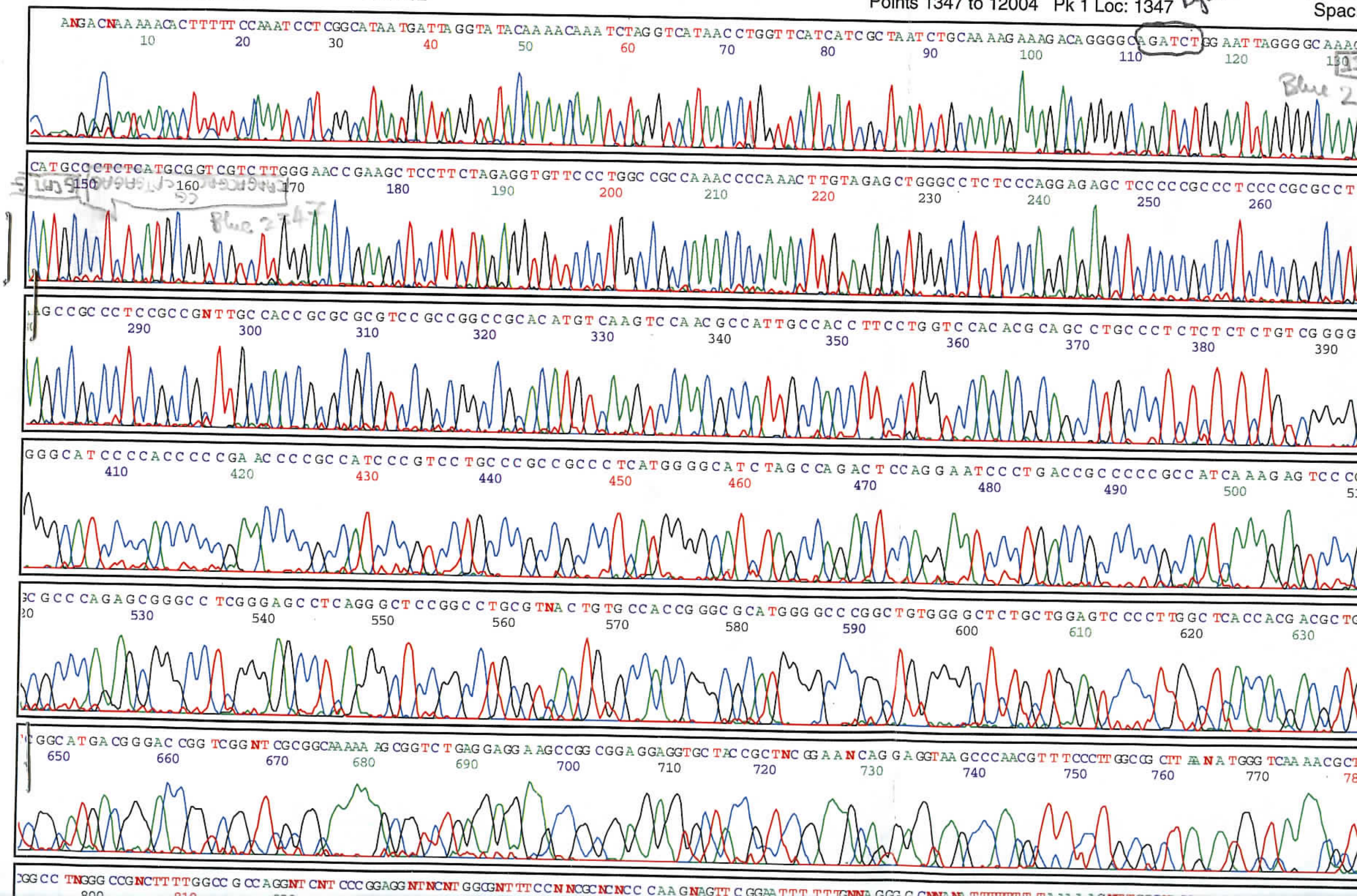
Model 377
Version 3.2
ABI200
Version 3.2

980815Ca52OSMI0814074

52OSMI0814074
Lane 52

Signal G:162 A:91 T:54 C:150
DT {BD Set Any-Primer}
96051547 DR LR9709 Instr File
Points 1347 to 12004 Pk 1 Loc: 1347 Bgl 2

Mon, Aug 17
Mon, Aug 1
Spac



On 3:55p
Off 4:25p
29 Hx1 Max

Fri. Aug 14th

PCR for Pgl2 — Exon 2

Dilution series again with DWM110 1/4 — 1/324 A ..... E

with 2.5+7.5 → 2.5+5 etc.

n=10

Blue Pfu
- ✓ 20x PCR 12.5
- ✓ ddw 187
- ✓ dNTPs 1
- ✓ DMSO 25
- ✓ H'69 Ref Seq Exon 2 5
- ✓ H'85 Blue 2357 primer 5

Black Taq
- 120 Blue 2.5 Pfu ////
- 120 Black 5 Taq ////

≥90 1 min
93-94 1 min
60° 78c
60° 5 min
35 cycles
On 11:28a

Acr MM
- 1 µl of A ... E
- 10 µl DWM 110 Buffer 4 Cla
- 10 µl DWM 110 Control
- W
- Dyt

Sat - Aug 15th

Took a chance & left at it. Sat. a.m.

OK

H'91 Seq #72	H'87 Mini #4 FAILED	1 µl ✓	each + 14 µl ddw
Primer H'85/2367 Blue ✓ x 1/100		5 µl ✓	
H'91 Seq #73	H'87 Mini #4	1 µl ✓	
Primer H'68 Red 1° x 1/100		5 µl ✓	
H'91 Seq #74	H'87 Mini #4	1 µl ✓	
Primer H'69 Exon 2 Silo Red ✓ x 1/100		5 µl	

Oligo # 19878
Date made:
LENGTH: 19
% G+C: 58%
5' Oligo Name: H'91/2347 Blue 3'

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

73 & 74 worked.
72 failed, ? due to 2° structure
Sequence is, however, ok.
∴ make new Blue primer

H'91 Seq #75	H'87 Mini #4	1 µl ✓ + 14 µl ddw
Primer H91 [redacted] / 2342 Blue ✓ x 1/100		5 µl ✓

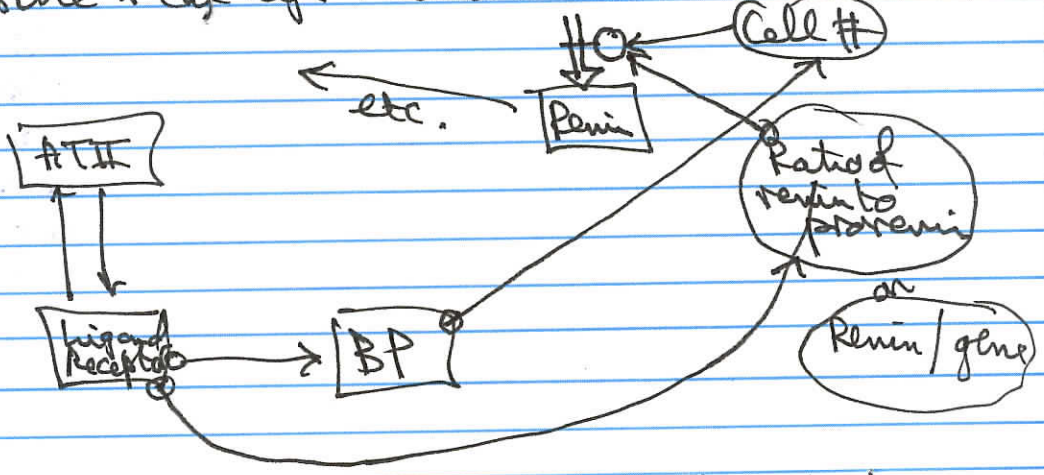
WORKED FINE

Sat. Aug 15th

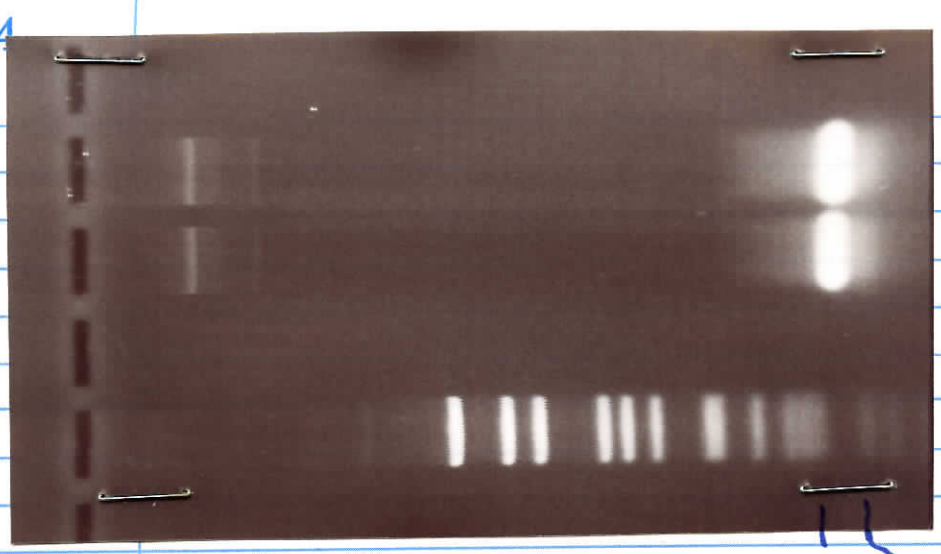
Stella Models

Masaki to visit at ~2 weeks ending Aug 16th
He demonstrated that ~~two~~ feedback loops
with the log$_2$ curve  or a curve (single) with log$_4$

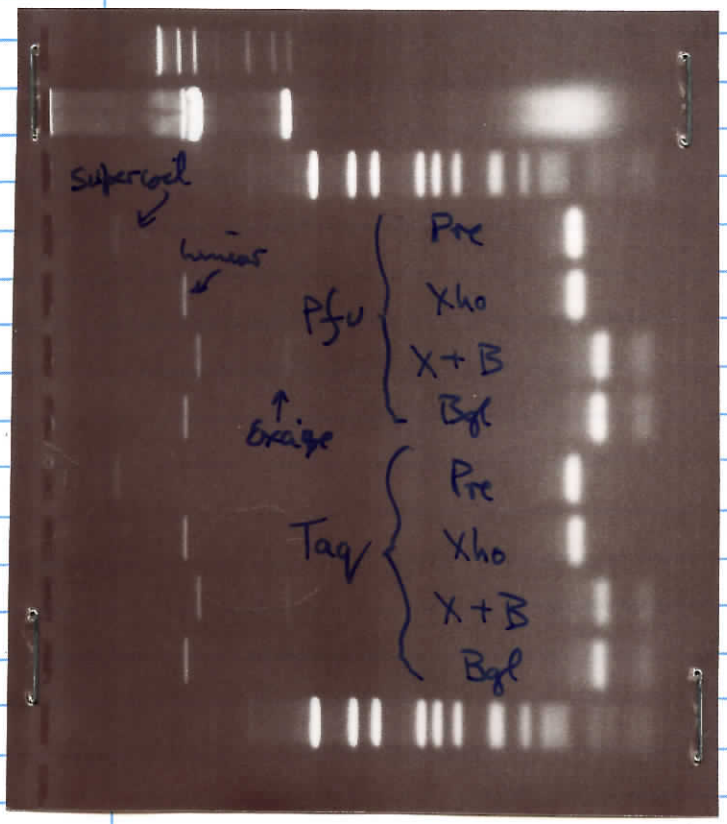
will reproduce the renin ~~feedback~~ better than old Feb 26th model.
The double feedback effect may (OS) be due to Stella ~~doing two~~ sequential corrections
⇒ so effectively squaring the effect.
Possible real life reason could be :—



A good case can be made for the stretch effects on vascular smooth muscle being ~~separate from~~ the effects of ATII although with both having a common final pathway in some cells.



① Conclusion
Excellent amplification.
Expected size is 210 bp
— agrees fine.



② Conclusion
All cuts successful → products are correct. Purify & proceed, but as rough try ligate unpurified Pfu digests.

32° 11

2347 Blue
H'87 #4 mini — 2°, 1° Red H'68

On 11:03 am
Off
1.5% HY1 Max

1
Dye
W
Pfu Blue H'95 PCR
Taq Black
W
ACR MM
W
Dye

On 4:42 pm
Off 5:19 pm
1.5% HY1 Max

2
Dye
W
PfG MM
Mini 4 Xho + Bgl
ACR MM
Pfu: Pre, Xho 1, Bgl 2 + Xho 1, Bgl 2
Taq: Pre, Xho 1, Bgl 2 + Xho 1, Bgl 2
ACR MM
W
Dye

Tues. Aug. 25th

Prep. PCR (a rough test)

n = 10

H'89	20 x PCR	12.5 μl	✓
	ddw	186 μl	✓
H'89	dNTP's	1 μl	✓
H'89	DMSO	25 μl	✓
H'68	Red 1° primer	5 μl	✓
H'68	Red 2° primer	5 μl	✓
H'87	#4 mini	1 μl	✓
H'91	2347 Blue primer	5 μl	✓

120 μl → 2.5 μl H'89 Pfu polymerase, 4 Blue tubes
120 μl → 5 μl H'89 Std. Taq, 4 Black tubes

≥ 90° ≥ 1 min
35 cycles: 93° 1 min; 40° 7 sec; 60° 5 min

6:50 pm Start

Thurs. Aug 27th

Both PCR ~~tubes~~ ppt'd with EtOH & back into 100 μl 0.001M EDTA as [H'95 Pfu] Blue and [H'95 Taq] Black → reppt'd to quire

Take		
✓ 20 μl 10 x R1 salts	✓ 22 μl 10 x R1 salts	✓ 22 μl 10 x R1 salts
✓ 170 μl ddw	✓ 158 μl ddw	✓ 158 μl ddw
✓ 10 μl H'87 #4 mini	✓ 20 μl H'95 Pfu	✓ 20 μl H'95 Taq

4 BX: ✓ 2 μl Bgl 2, ✓ 1 μl Xho 1

Pfu: 18 μl → P Pre; 100 μl + 2 μl Bgl 2 → 92 / 92 → P B, P BX; 100 μl + 1 μl Xho → P X

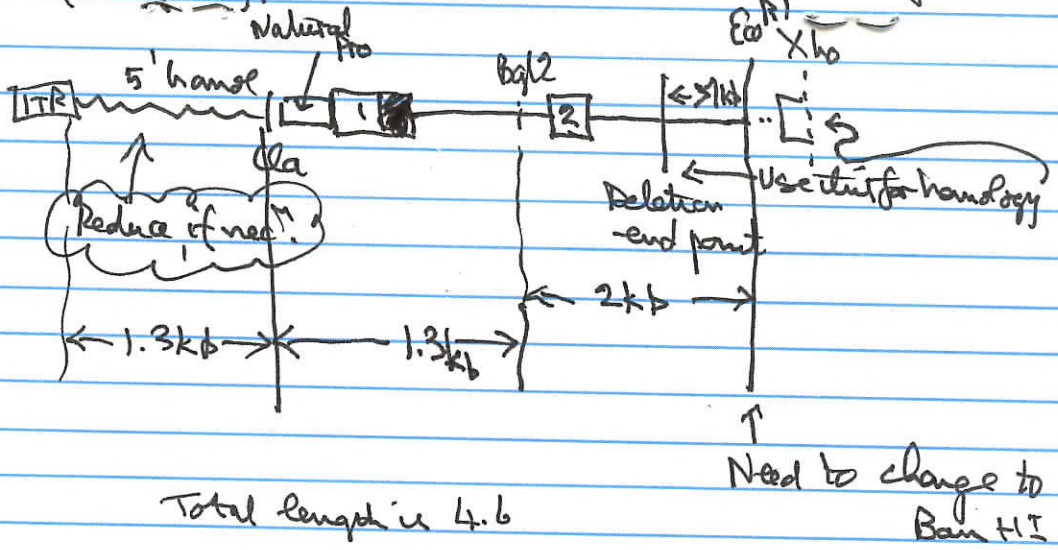
Taq: 18 μl → T Pre; 100 μl + 2 μl Bgl 2 (lot 36, 10 u/μl) → 92 / 92 → T B, T BX; 100 μl + 1 μl Xho I (lot 35, 20 u/μl) → T X

37° 11:45 am

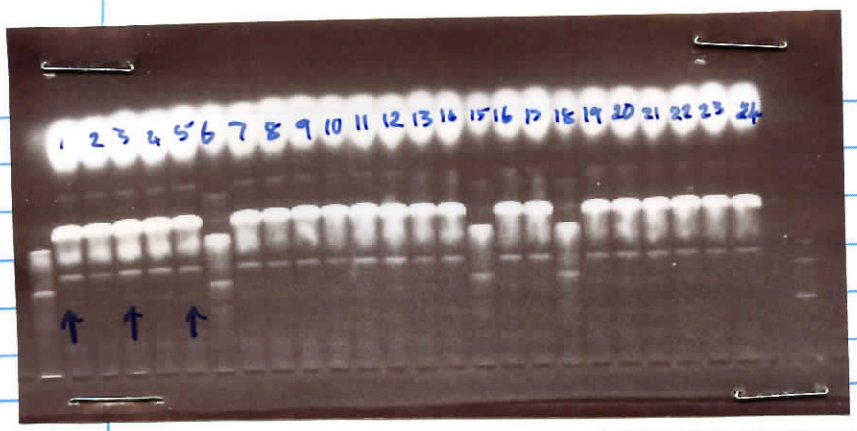
Thurs. Aug 27th

H.R. construct – redesign

Seiko suggested trying to keep intron 2 of HPRT by using pMP8. In turn Jeff suggests following from pSVM 11b (modified):–



Total length is 4.6



D

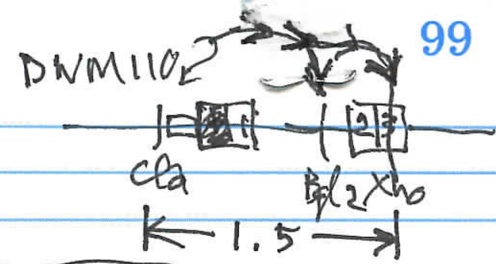
Conclusion: Looks like 21/24 !
Proceed with
(1) (3) & (5)

(1)

1+14

① 10:15 am ~~off~~ 0.86 Hx4

Fri
~~Thurs.~~ Aug 28th



Bgl
1-14 H'87 #4 mini

Ligation (rough)

2 H'99 minis

~~Take 10μl H'95 4BX (Mini) 5μl H'95 P(...) BX Heat 90~~

23
24
W
PGMM
W
Bgl

Blue Black
H'95 PBX, TBX & 4BX φOH/HOQ, 2xCIAA

Take ✓ 8μl H'95 4BX · Bgl2/Xho1 of mini #4
✓ +20μl H'95 PBX PCR product after Bgl2/Xho1
✓ +3μl 8M NH_4Ac
+78μl EtOH
Freeze, Cent, dry → back into 30μl ~~0.001M EDTA~~ 1x p95 lig^n buffer
~~as H'99 MIX~~

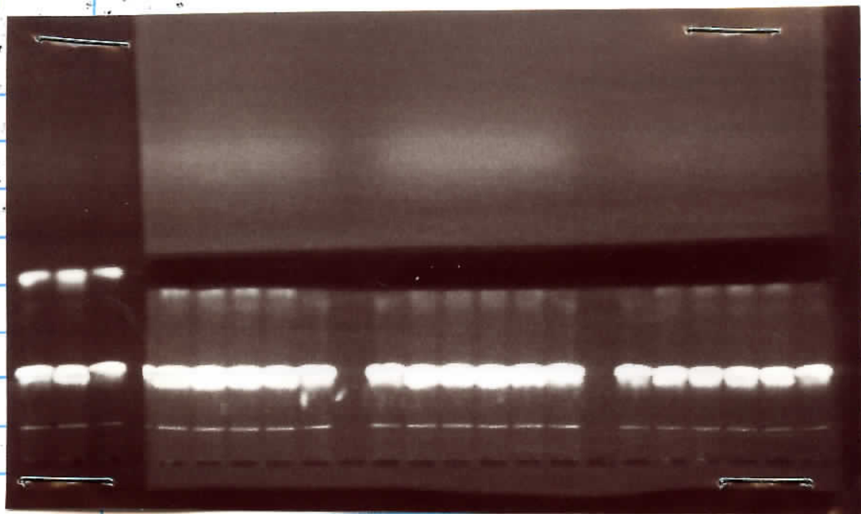
✓ 20μl H'99 Mix
~~+3.3μl 10x p95 buffer~~ lig. — H'99 LIG^N
✓ +2μl T4 ligase Dilute Lot 67
10μl + 8μl — H'99PRE
15° at 10am to 12:20pm RT → 5:30pm → Gel

H'35 DH5α	80μl
H'99 LIG^N	5μl
0° 5:32pm to 6:15pm	
90 sec	42°
Plated Rest 20, 5, 20/16, 5/16 1xAmp NZY	37° 6:25pm to 10:45am → RT

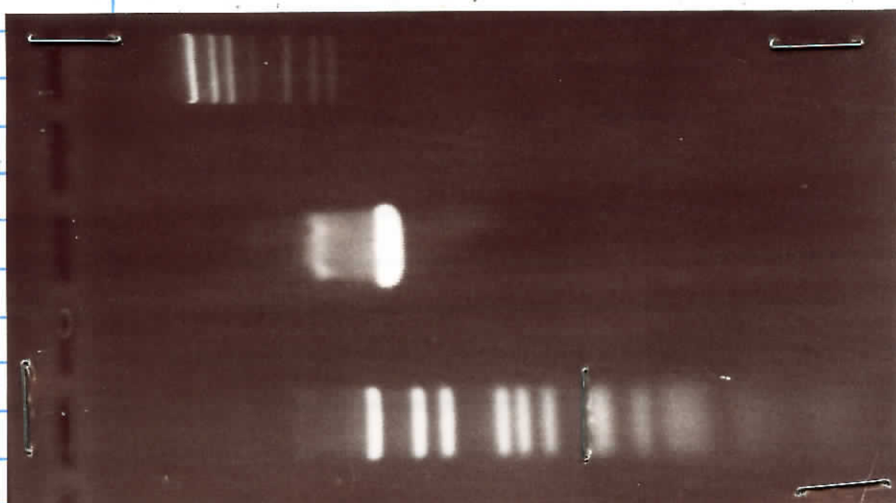
(√33)

1-24 # H'99 minis 37° 6:30pm fat
1-2 x Amp NZY

Processed H'99 minis ①, ③ + ⑤



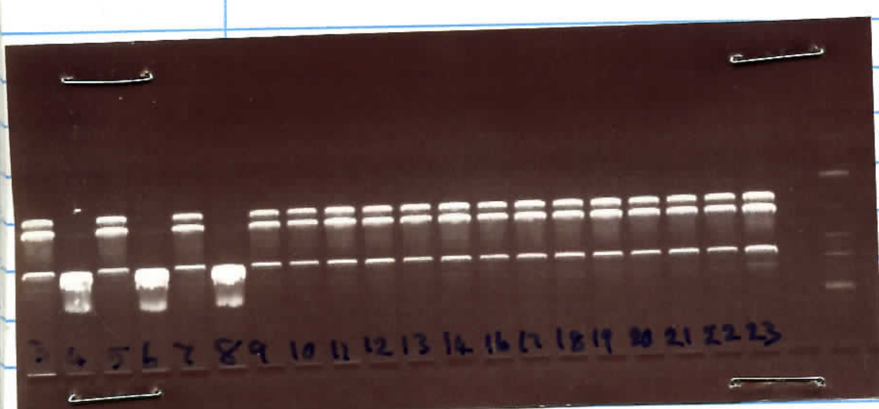
(1) Good safe cut.



(2) Yield terrific
Purity? ok?

Mon Nov 23rd 2:15 pm [illegible]

(3) See H'103



H'102 gel

(see H'102 also)

(3) Conclusion:
4, 6 & 8 have lost
one Sma I site; others
are okay & have both present

Linear ~ 8 kb } 7.9
~~insert~~ ~~vector~~ 4.3 kb } 4.35
Vector ~~insert~~ ~3.8 kb } 3.7

Check sizes again

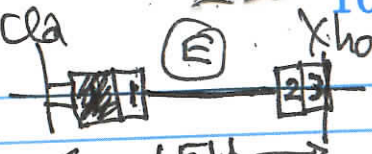
Correct

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

(4) Conclusion: All the plasmids show same instability & lose an ITR — but can proceed.

Sun. Aug 30th

Fragment (E)



Take ✓ 30µl 10× Eco RI salts
✓ 221µl ddw
4µl Xho I Lot 35/20u/µl ≡ 80/15 u/20µl ~5
✓ 15µl Cla I Lot 33/5u/µ-µ ≡ 5u/20µl

90 ✓ 90 ✓ 90 ✓

+10µl H'99 (1) H'99 (3) H'99 (5)

37° 1:57 pm — 5:22 pm

Back into 100µl as H'101 Frag: E c/x

To miscellaneous

Tues. Nov 24th

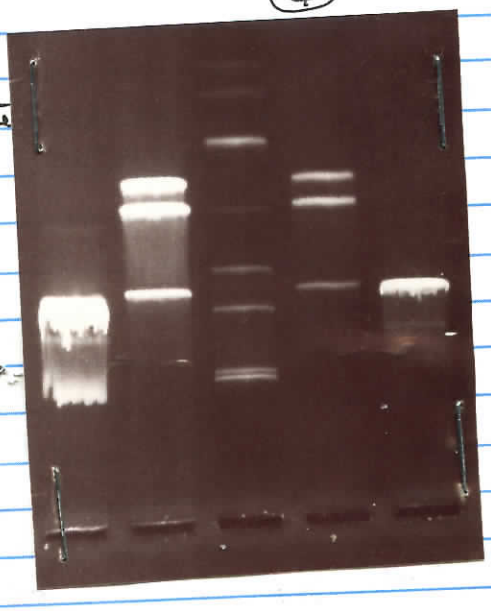
On 10am Off 11:20 ~

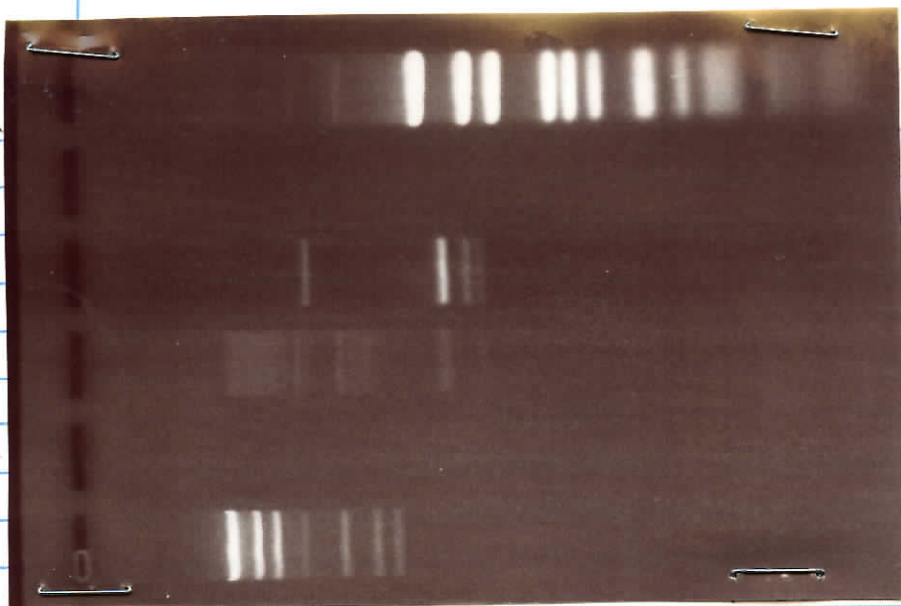
0.8% HxL4R

(4)

4+6+8 SmaI overnite
18-23 " "
AGMM
(B)/5
(A)/5
W
Dye

(A)
(B) Extra SmaI

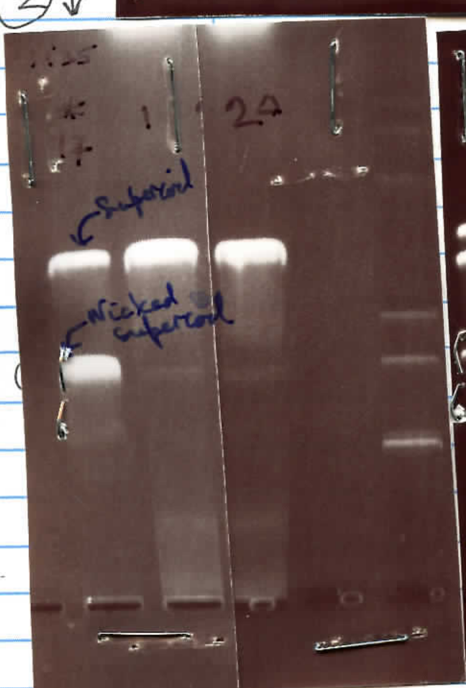




① Good ligation but could have used ½ X frag^t E[?]

② Either all carried on[?] !

②↓



② Higher magnific.

Nov. 19th, 1998

Seigo found that #15 had lost ability to be excized with cuts in the ITR's. He will test #1 & others. If a problem may have to go back to Vector SSV prep?

Nov. 21st, 1998

Seigo found that #1 was fine & cuts with Sma I. Seigo also tested #2, #23 → #24 — OK

Processed remainder to 50 µl

From H'100 ③

Gel on H'100

Nov. 23rd   40 µl NEB buffer 4 10X
350 µl dd H₂O

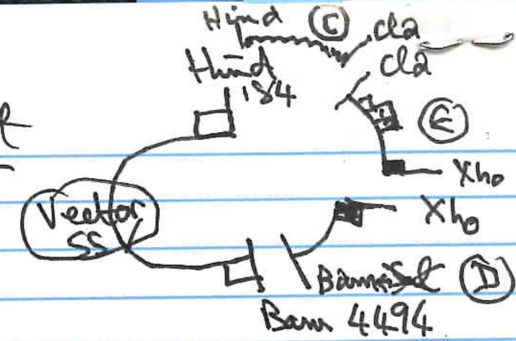
1 µg of new maxi prep of 23   10 µl Sma I Lot 52 100/µl

R.T. from 11am to 1:40pm 37° 1:40pm to 2pm (½ life 15 min at 37°)

On 10:40 Off 11:50 0.8% H×4 MAX

Mon. Aug 31st

Assembly



MAX λ 1.5% H× Ⓘ H'101 DWMMod(ified)

On 1:20 Off 1:50pm

Take

Phenol !	✓ 2.5 μl SSV H/B	H'33 conc (Vector)
1) Age W	✓ 10 μl Frag.t Ⓒ	H'1 conc
H'35#1 AGR MM	✓ 2 μl Frag.t Ⓔ	H'101 C/X
H'103#1 W	✓ 10 μl Frag.t Ⓓ	X to B H'81
2 Pre H'103	✓ 2.5 μl 5M NH_4Ac	
W Post H'103	✓ 67.5 μl EtOH	
AGR MM		
23 W		
24 Phenol !		
W		
AGMM		
W		
Dye		

Into 10μl 1x p95 lig.n buffer
+ 0.8μl T4 ligase dilute lot 67 — H'103 LIG.N

2.5 to 12.5 — Pre H'103

Cold room 5:30 pm to 1:10pm → to cold

2.5 to 12.5 — Post H'103

Transformation

H'35 DH5α | 80μl

2.5μl ← ~3pm

0° ~3pm to ~5pm

90sec. 42°

Plated 1x Amp NZY — Rest, 20, 5, 20 / 16, 5 / 16 — 37° ~5pm

Many ⑫ ①

H'103 minis #1–24 Thurs.

1x Amp NZY 37° 6:05pm

Procelled #1 ~~[illegible]~~ #15

To Seigo as H'103#1 → Renamed HR-3

H'103#15

Pooled rest of digest of #18–23 + 10u (1μl) Sma I ~~at room~~ at R.T. 6:35pm

Run more dilute in longer gel.

{ H/B Vector 4.3 kb

15HB

PCR MM

DWM. cons. Mini 15 H'03 H'68 sorted

```
1650 GCTGAGGCGGCGAGGGAGAGCGTTGGGCTTACCTCACTGCTTTCCGGAGC 1699
756  .CTNA.GC.GCAAAGGANAG.GTT.GGCCTA.CTCA.TGCTTTCCGGAGC 714

1700 GGTAGCACCTCCTCCGCCGGCTTCCTCCTC.AGACCGCTTTTTGCCGCGA 1748
713  GG.AGCACCTCCTCCGCCGGGTTCCTCCTCAAGACCGCTTTTTGCCGCGA 665

1749 GCCGACCGGTCCCGTCATGCCGACCCGCAGTCCCAGCGtc.TG.TGAGCC 1796
664  GCCGACCGGTCCCGTCATGCCGACCCGCAGTCCCAGCGTCGTGGT.AGCC 616

1797 AAGGGGACTCCAGCAGAGCCCCACAGCCGGGCCCCATGCGCCCGGTGGCA 1846
615  AAGGGGACTCCAGCAGAGCCCCACAGCCGGGCCCCATGCGCCCGGTGGCA 566

1847 CAGTgACGCAGGCCGGAGCCCTGAGGCTCCCGAGGCCCGCTCTGGGCGCT 1896
565  CAGTGACGCAGGCCGGAGCCCTGAGGCTCCCGAGGCCCGCTCTGGGCGCT 516

1897 GGGGCGGCCGGGACTCTTTGATGGCGGGGGCGGTCAGGGATTCCTGGAGT 1946
515  GGGGCGGCCGGGACTCTTTGATGGCGGGGGCGGTCAGGGATTCCTGGAGT 466

1947 CTGGCTAGATGCCCCATGAGGGCGGCGGGCAGGACGGGATGGCGGGGTTC 1996
465  CTGGCTAGATGCCCCATGAGGGCGGCGGGCAGGACGGGATGGCGGGGTTC 416

1997 GGGGGTGGGGATGCCCCTCCAGTGAGCCCCGACAGAGAGAGAGGGCAGGC 2046
415  GGGGGTGGGGATGCCCCTCCAGTGAGCCCCGACAGAGAGAGAGGGCAGGC 366

2047 TGCGTGTGGACCAGGAAGGTGGCAATGGCGTTGGACTTGACATGTGCGGC 2096
365  TGCGTGTGGACCAGGAAGGTGGCAATGGCGTTGGACTTGACATGTGCGGC 316

2097 CGGCGGACGCGCGCGGTGGCAAgCGGCGGAGGGCGGCTTCAGGAAGGATC 2146
315  CGGCGGACGCGCGCGGTGGCAAGCGGCGGAGGGCGGCTTCAGGAAGGATC 266

2147 AGGCGCGGGGAGGGCGGGGGAGCTCTCCTGGAGAGGCCCAGCTCTACAA 2196
265  AGGCGCGGGGAGGGCGGGGGAGCTCTCCTGGAGAGGCCCAGCTCTACAA 216

2197 GTTTGGGGTTTGGCGGCCAGGGAACACCTCTAGAAGGAGCTTCGGTTCCC 2246
215  GTTTGGGGTTTGGCGGCCAGGGAACACCTCTAGAAGGAGCTTCGGTTCCC 166

2247 AAGACGACCGCATGAGaggGCATgTTCAGGCCTGGACTTTGCCCCTAATT 2296
165  AAGACGACCGCATGAGAGGGCATGTTCAGGCCTGGACTTTGCCCCTAATT 116

2297 CCAGATCTGCCCCTGTCTTTCTTTTGCAGATTAgCGATGATGAACCAGGT 2346
115  CCAGATCTGCCCCTGTCTTTCTTTTGCAGATTAGCGATGATGAACCAGGT 66

2347 TATGACCTAGATTTGTTTTGtATACCTAATCATTATGCCGAGGATTTGGA 2396
65   TATGACCTAGATTTGTTTTGTATACCTAATCATTATGCCGAGGA.TTNGA 17

2397 AAAAGTGTTTATTCCTCATGGACTGATTATgGACAGGTTAGTAAGACCTC 2446
16   AAAAGTGTTT.TTNGTC..........78 seq.............. 1
```

8Cb15OSMI0904076

MI0904076

15

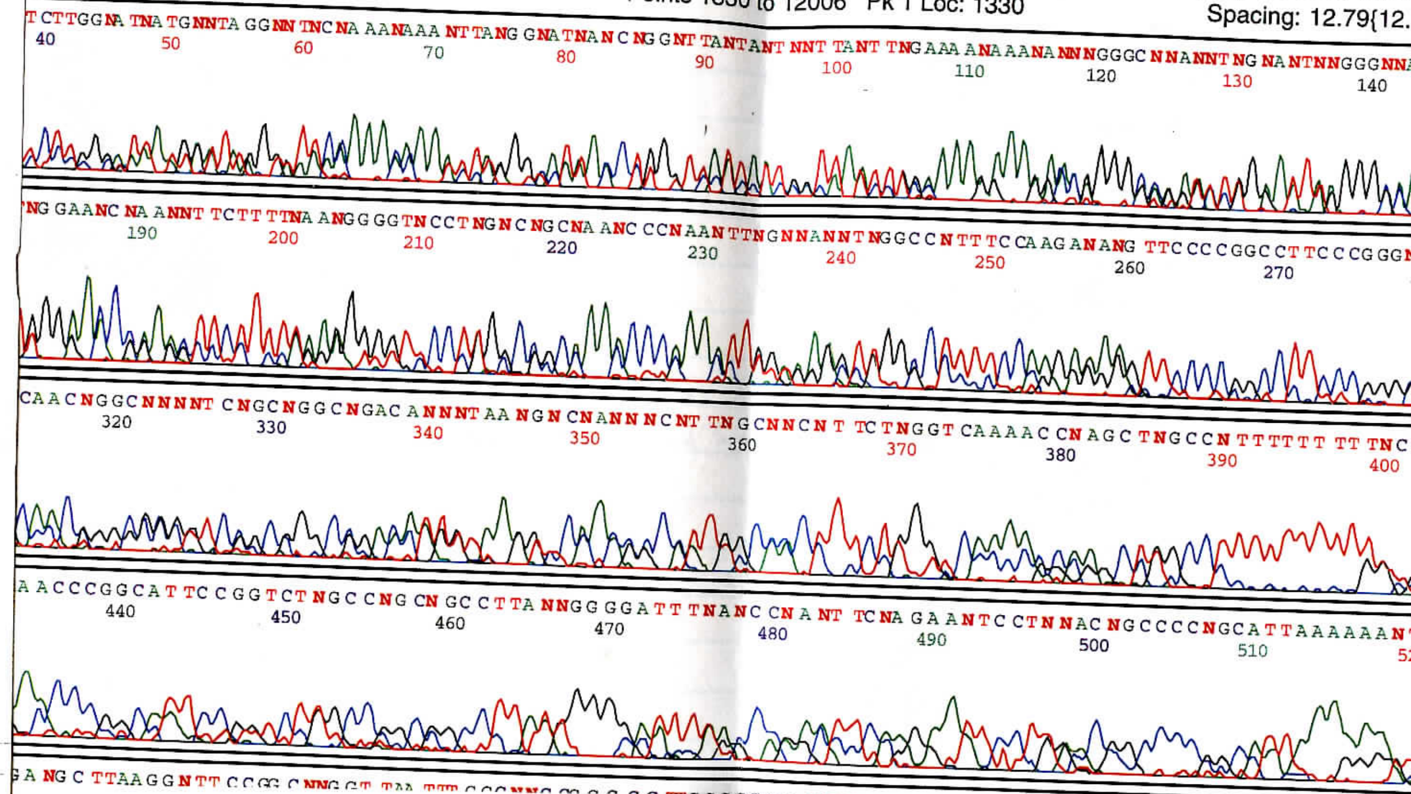
Mini 15

PE123[illegible]I1

H'68 Red 2° primer (in exon 3)

Signal G:164 A:96 T:54 C:130
DT {BD Set Any-Primer}
96051547 DR LR9709 Instr File
Points 1330 to 12006 Pk 1 Loc: 1330

Page 1
Tue, Sep 08, 1998 11:59
Tue, Sep 08, 1998 5:13
Spacing: 12.79{12.



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

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

TC TTTT C EAAAA T CCTC G G AATAA TGATTAG G T A TN CAAAACAAAA TCT AG 18p. EA

Second sequence

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

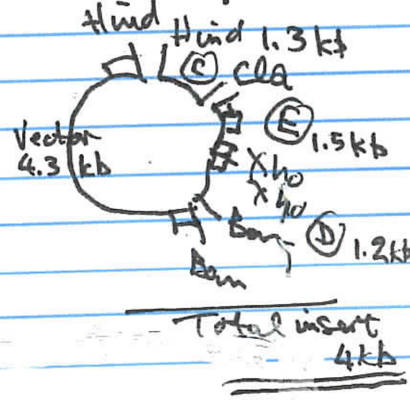
Dye
AgMM
1HB
15HB
ACR MM
1HC
15HC
1CX
15CX
1BX
15BX
H'101 Minis
Dye

Thurs. Sep. 3rd

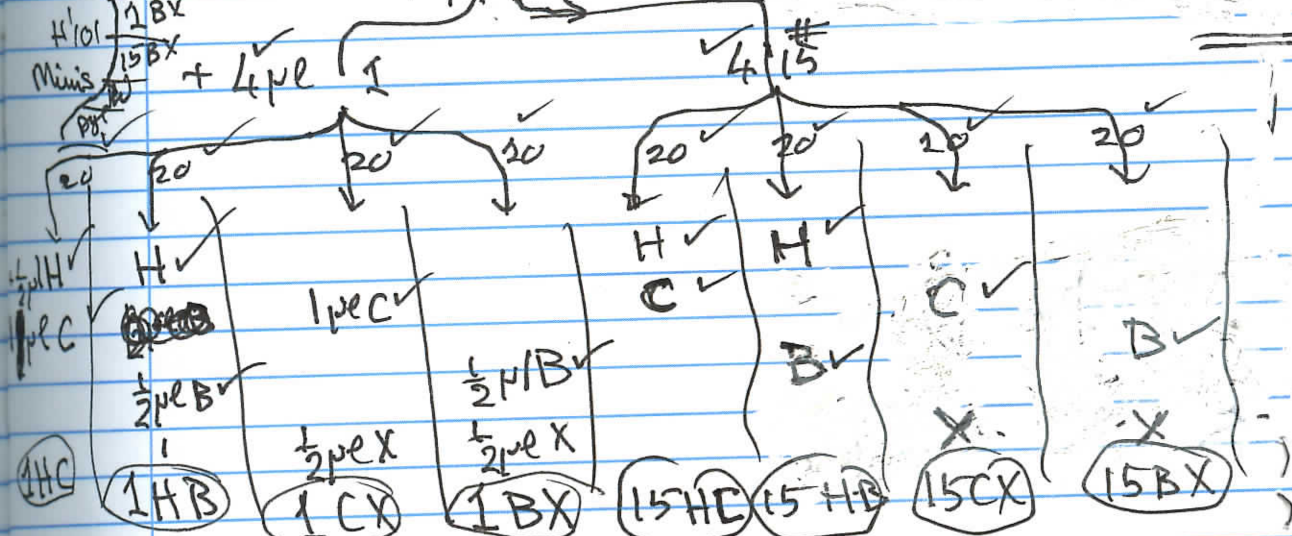
WW2 #1!

H'101 = #1 & #15

Check on candidates



Take 8 µl 10×R1 salts | 2×
72 µl ddw



Cla lot 35 20u/µl Hind 3 lot 50 20u/µl Cla 1 Lot 33 5u/µl Bam H1 lot 77 20u/µl

37° 1:40pm – 3:05pm

Streaked out H'103 #15 & named it PE(xon)123 I(ntron)1 (promoter)

H'103 **PE123 I1** → Renamed → **HR-3**

#1 streak out Sep. 10th

Sequence check Seq. #76 H'103 Mini #15 1µl ✓
Ddw 14µl ✓
H'68 Red 2° [scribbled out] Primer/100 5µl

see page 106

Results confirms assembly – but a badly mixed sequence – one base pair out of synch.
Try again with H'103 #1 and with a different primer – probably at 6T

Same problem probably primer

PE1 2(1) Sep. 10th

Seq. #77 H'103 mini #1 1µl ✓
ddw 14µl ✓
Primer/100 H'68 Red 2° 1/100 5µl ✓

Seq. #78 PE1 2(15) OK >700bp
H'103 mini #15 1µl ✓
14µl ✓
H'68 Solo Red Exon 2 1/100 5µl ✓

ABI PRISM™

Model 377
Version 3.2
ABI200
Version 3.2

980911Db51OSMI910001

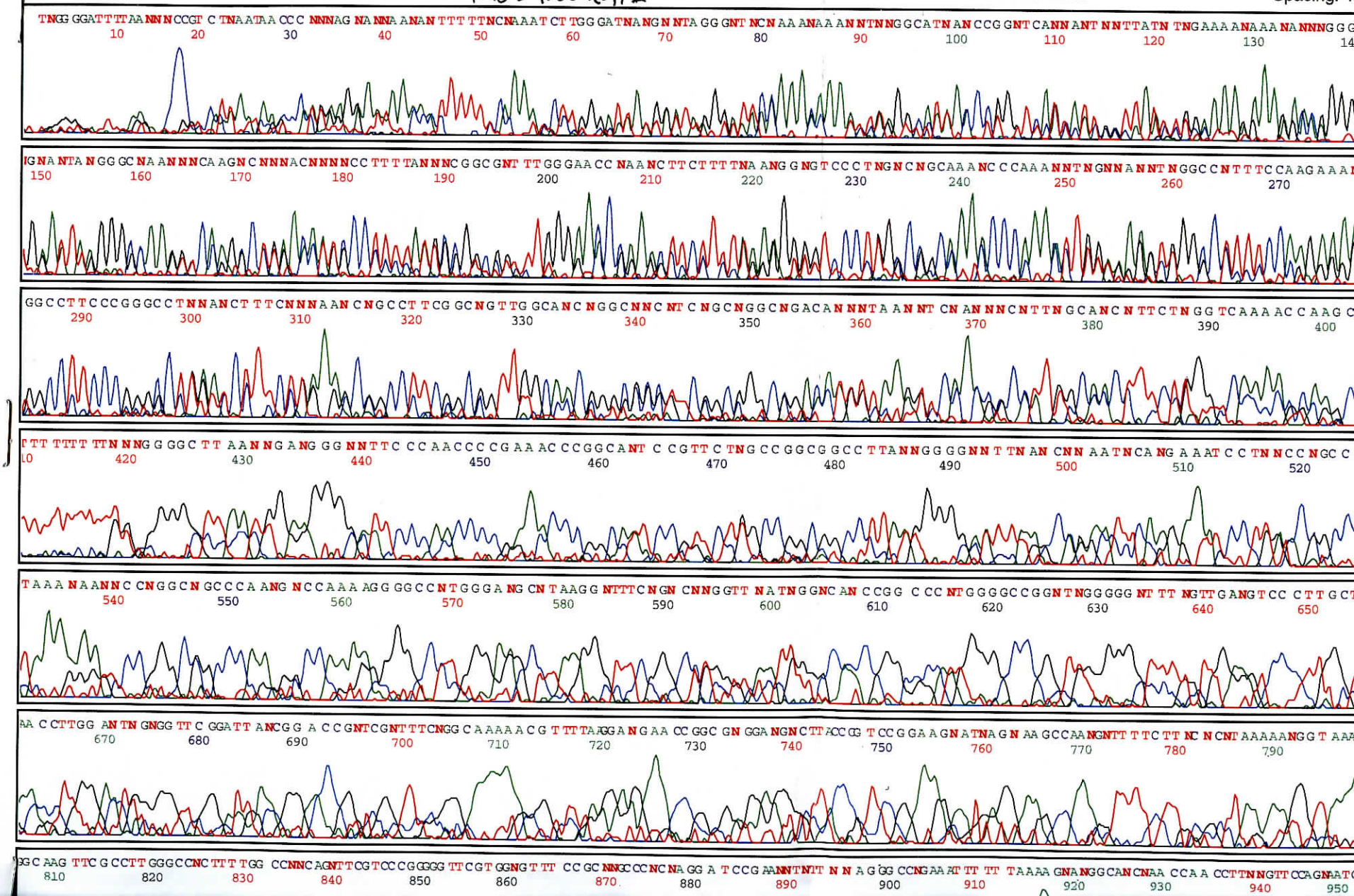
51OSMI910001
Lane 51

Colony 1 PE123N1
H'68 Red 2° primer
H'108 Mini #1

Signal G:87 A:48 T:29 C:76
DT {BD Set Any-Primer}
97022467 DR LR9710a Instr File
Points 1220 to 12006 Pk 1 Loc: 1220

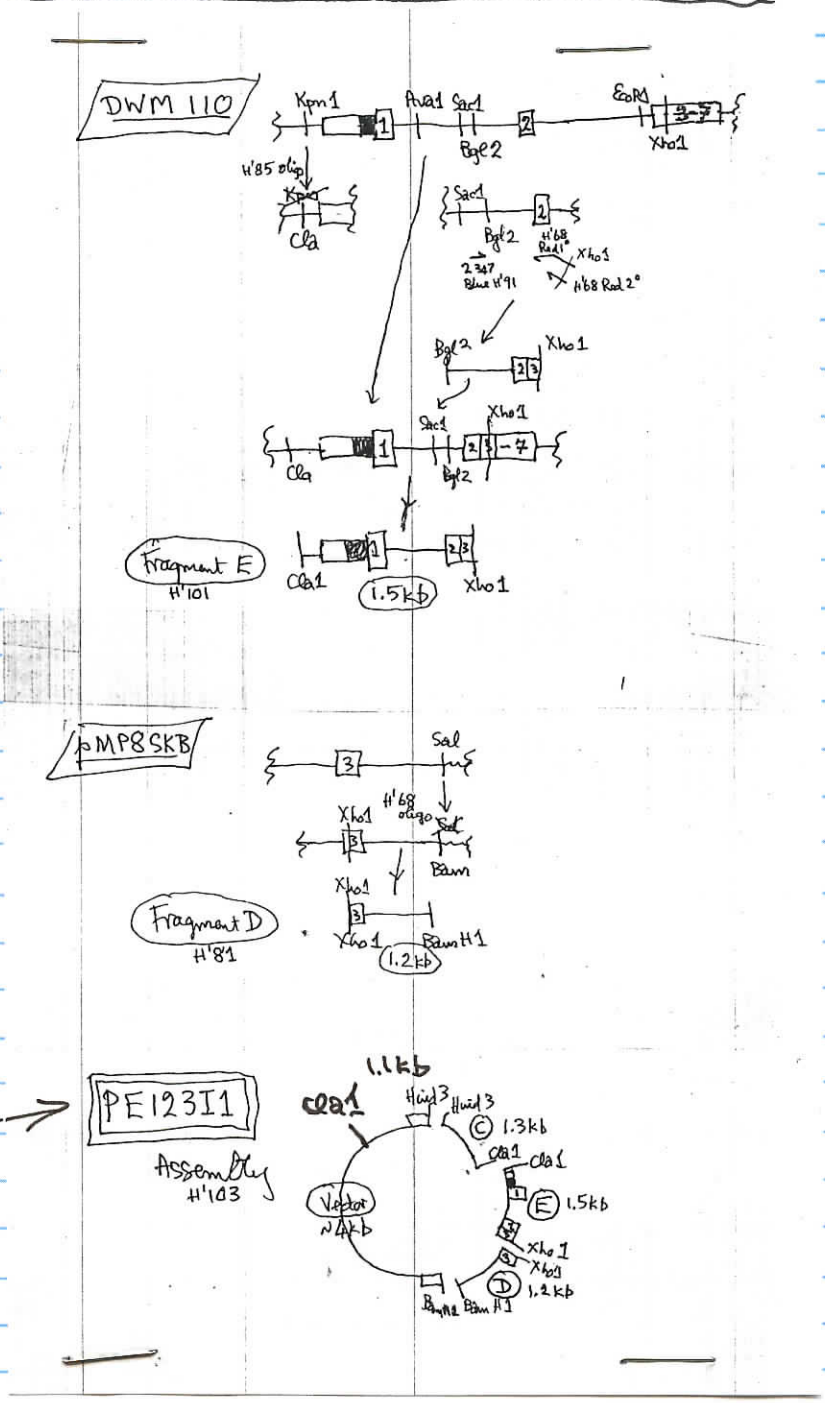
77. Seq.

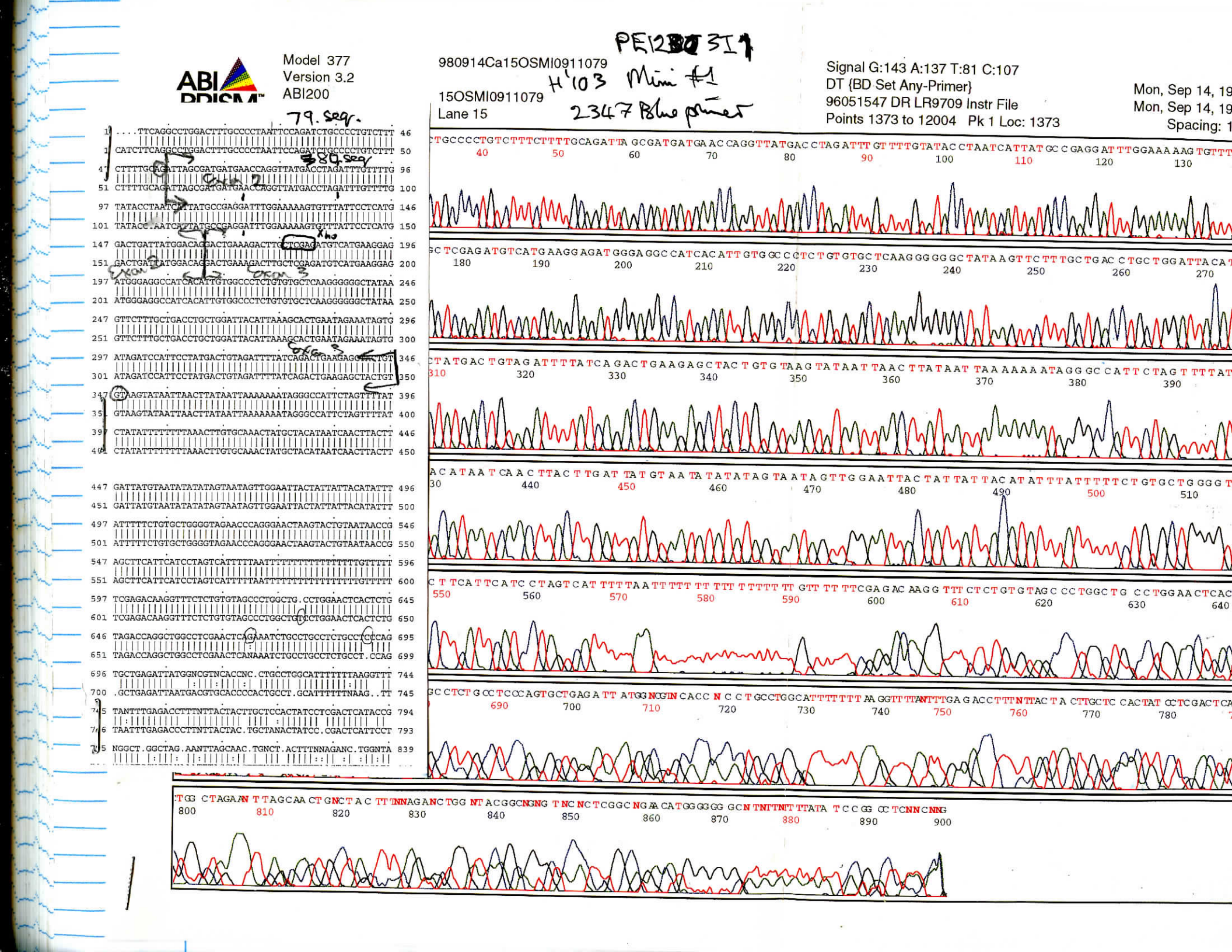
Mon, Sep 14
Fri, Sep 11,
Spacing: 1



Fri. Sep. 4th → Assembled and annotated as HR3.seq Jan. 30th, 1999.

PE123I1 Summary & sequence check




Model 377
Version 3.2
ABI200
980914Ca15OSMI0911079
15OSMI0911079
Lane 15
Signal G:143 A:137 T:81 C:107
DT {BD Set Any-Primer}
96051547 DR LR9709 Instr File
Points 1373 to 12004 Pk 1 Loc: 1373
Mon, Sep 14, 19
Mon, Sep 14, 19
Spacing: 1
79. seq.
Exon 2
Exon 3
Xho

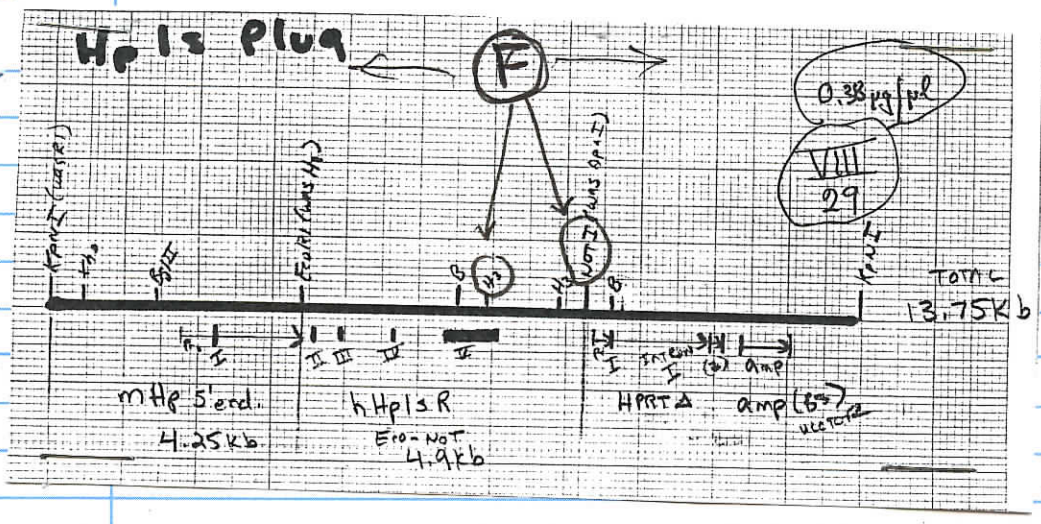
Fri. Sep. 11th

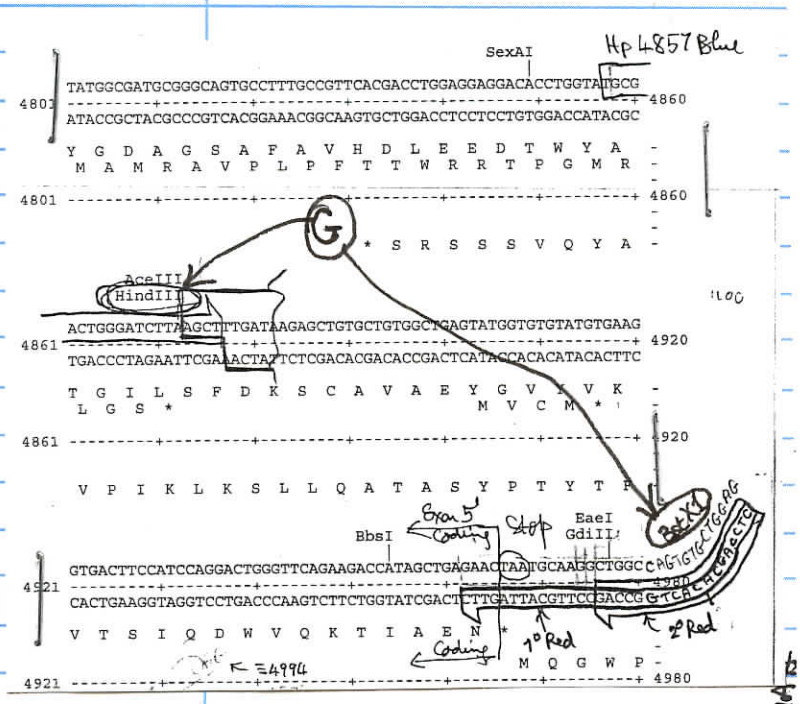
More PE123I1 sequencing

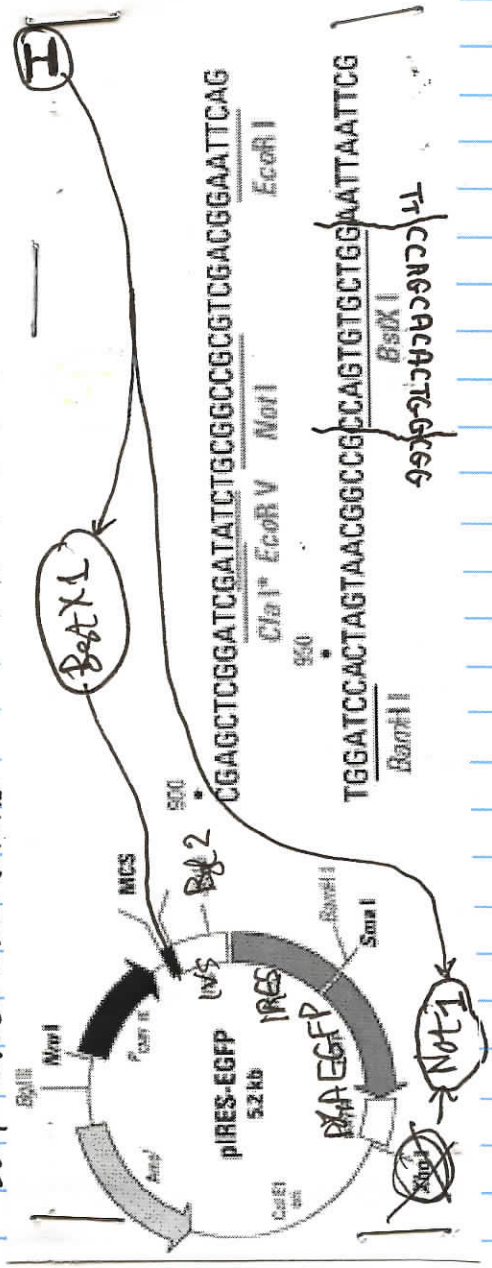
H'91 (see H'76) Blue direction sequencing worked well with Blue primer 2347. ∴ set up:—

Seq #79		Seq. #80	
H'103 mini #1	1 µl	H'103 mini #15	1 µl
DdW	14 µl		14 µl
1/100 H'91	2347 Blue Primer 5 µl		5 µl

Conclusion Both agree. The problem was the primer not the clones.







Oligo # 20053 5' Oligo Name: Hp4857Blue 3'
Date made: TGC GAC TGG GAT CTT
LENGTH: 20 AAG CT
% G+C: 50%

Oligo # 20054 5' Oligo Name: HpEx5 Red 1° 3'
Date made: CTC CAG CAC ACT GGC
LENGTH: 32 CAG CCT TGC ATT AGT
% G+C: 56 TC
Purify this one

Oligo # 20055 5' Oligo Name: HpEx5 Red 2° 3'
Date made: CTC CAG CAC ACT GGC
LENGTH: 18 CAG
% G+C: 67%

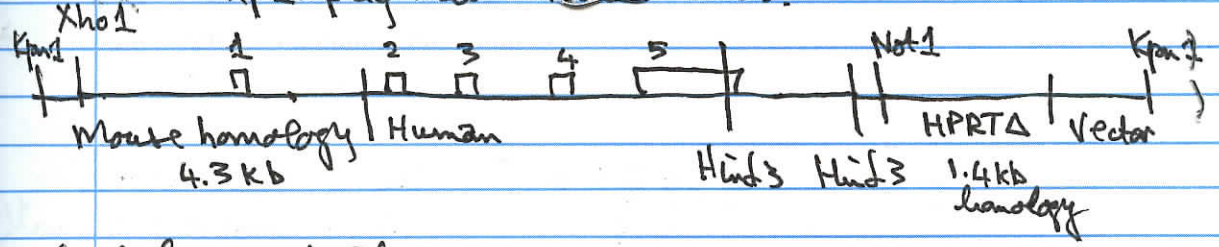
STORE @ -20 IN MEDIA LYSIS

Fri - Sep. 11th

Thoughts on Hp -/-

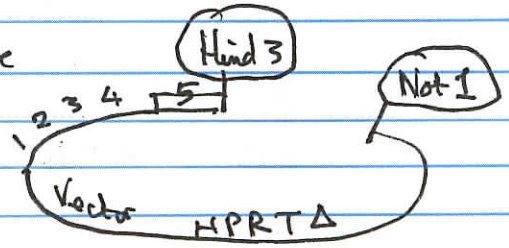
Rauty T- has many targetted clones in several ES cell lines that give Hp disruption. None go germline → no agouti are born. Chimeras are smaller than normal → have some quantitative peculiarities.
∴ worth finding where Hp is expressed without knocking out the gene. Look to see if Rauty's Huttp18 plug can be converted to Hp1S IRES GFP pA.

Hp1S plug has 2 Hind3 sites:—

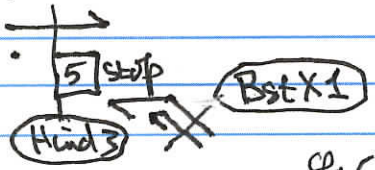


Can be linearized with Ase1 in vector, or (o.s.?) Xho1 in mouse homology or (?) Kpn.

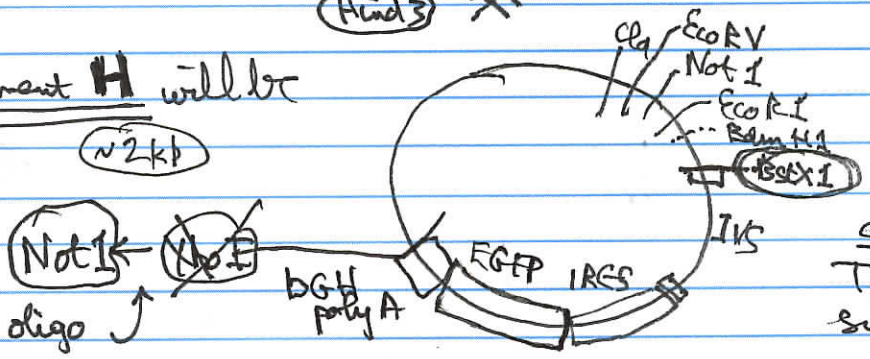
Fragment **F** will be ~12kb



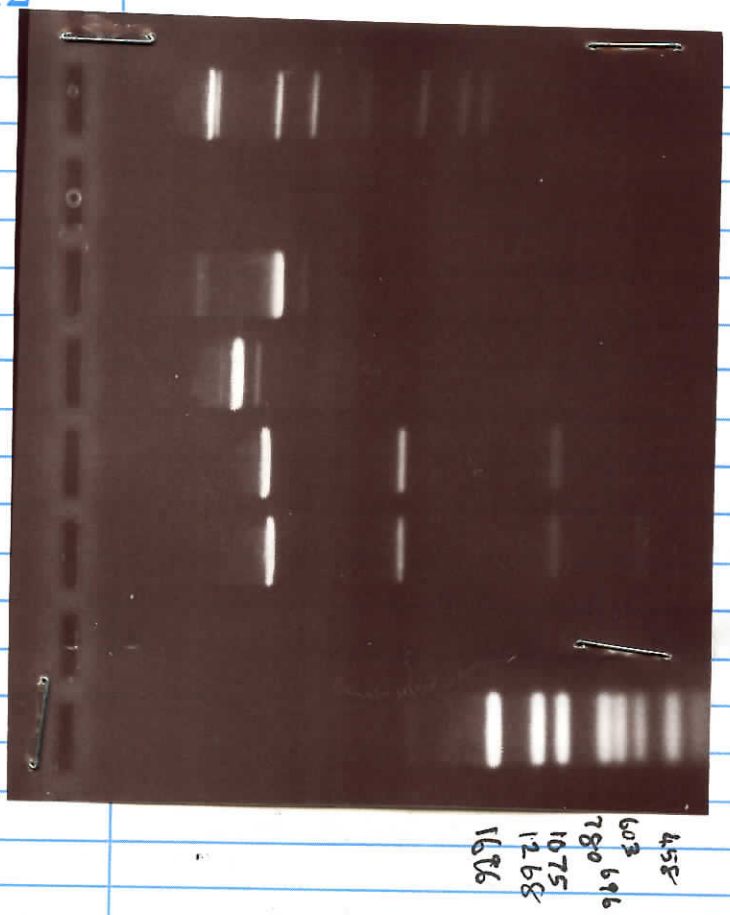
Fragment **G** will be by PCR 115bp



Fragment **H** will be ~2kb

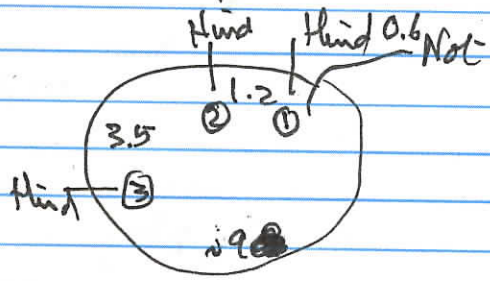


CAUTION! There is a KpnI site in EGFP.



① Construct 4'd mutated a Hind 3 site in Randy's gel (photo copy was too dark).

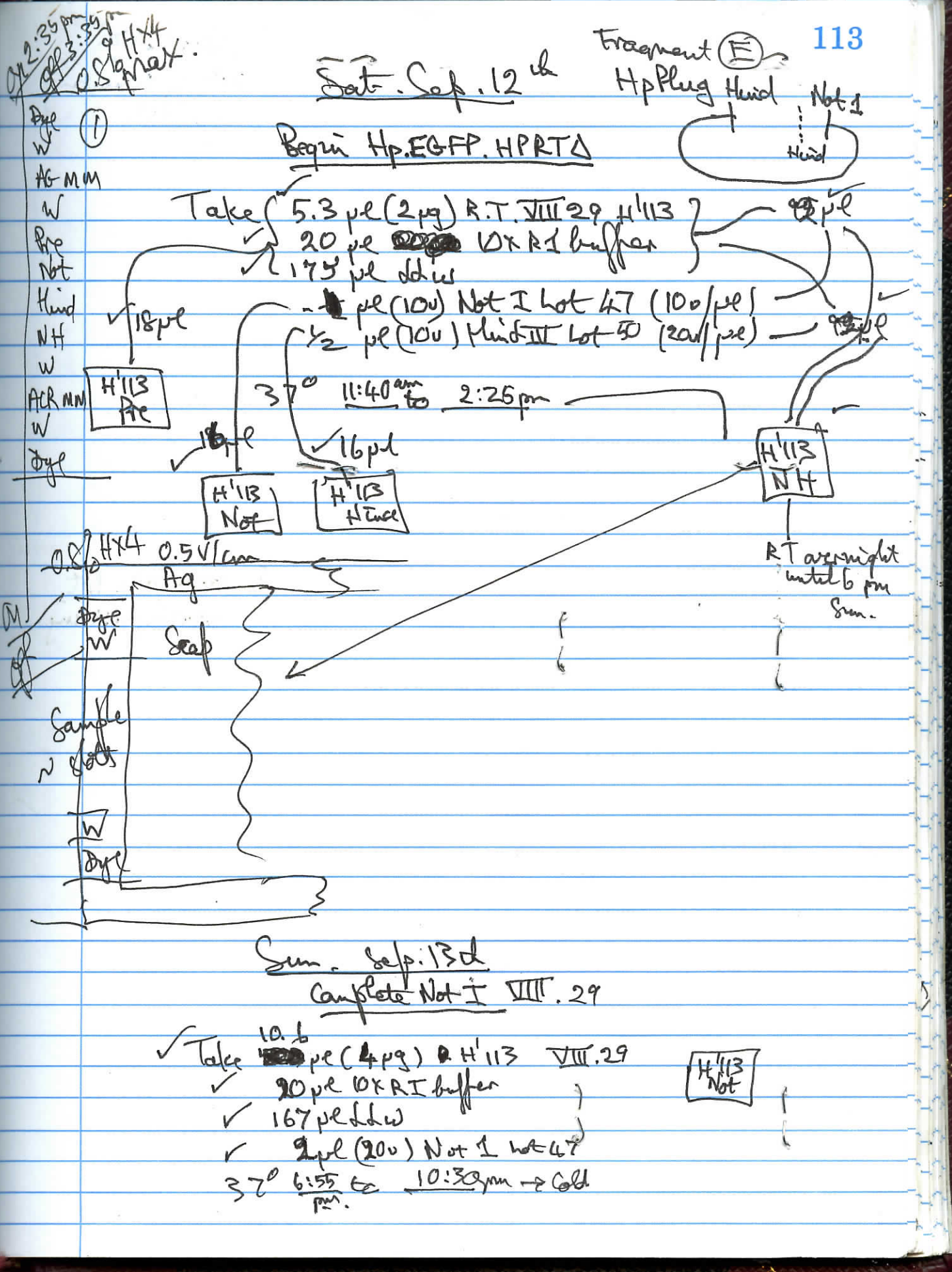
Map is: —

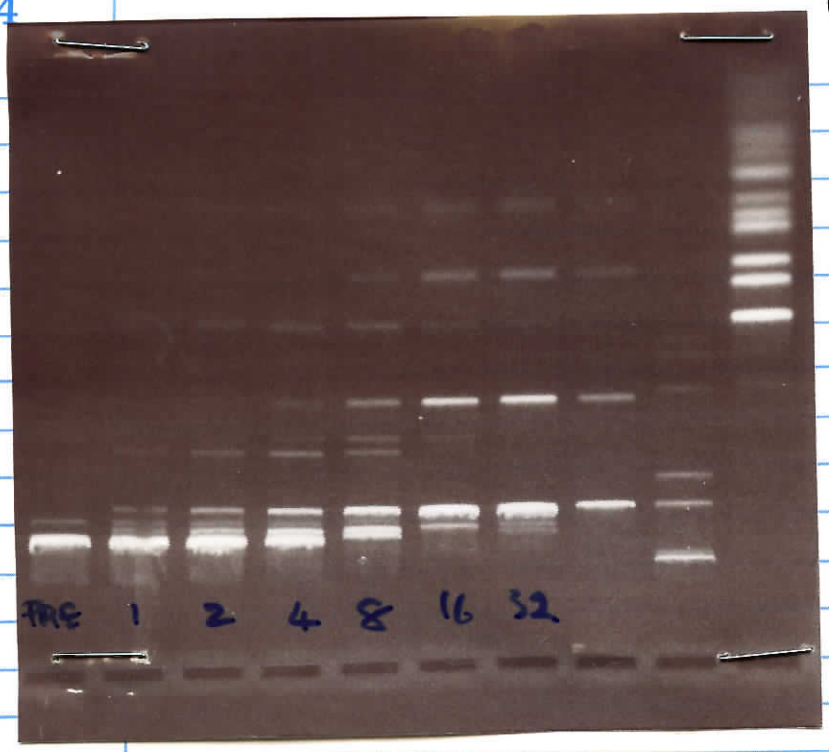


Need to rethink ? go with single cut Hind 3, & then complete cut Not 1 to get fragment ⑤ One of extras will be 1.2kb longer & one will be 3.5 kb shorter.

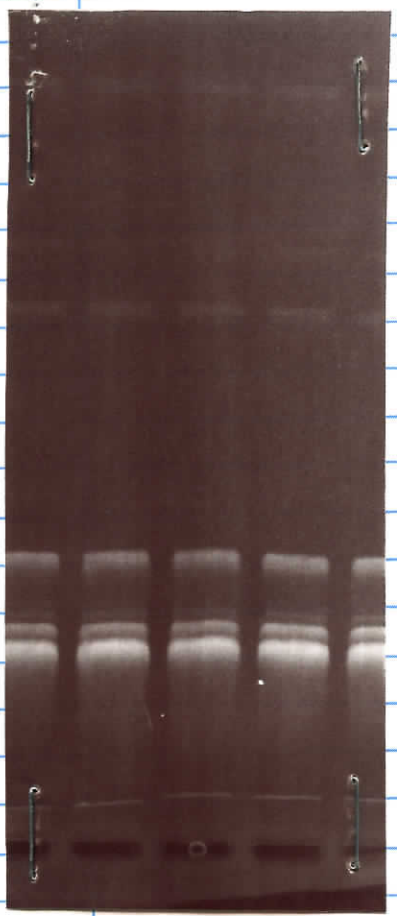
Better may be to do Not 1 first since sizes are better :—

	14 kb	Not 1 alone
	13.5 kb	Hind ① Not
Desired →	~ 12 kb	Hind ② (± ①) Not
Complete	~ 9 kb	Hind ③ (± ① ± ②) Not


On 2:35 pm
Off 3:35 pm
0.8% HX4 0.5 V/cm max.
Dye
W
AG MM
W
Pre
Not
Hind
NH
W
ACR MM
W
Dye
Sat. Sep. 12th
Begin Hp.EGFP.HPRTΔ
Fragment E
HpPlug Hind
Not 1
Hind
Take
5.3 µl (2 µg) R.T. VIII 29 H'113
20 µl 10x R1 buffer
175 µl ddw
µl (10u) Not I Lot 47 (10u/µl)
½ µl (10u) Hind III Lot 50 (20u/µl)
µl
18µl
H'113 Pre
37° 11:40 am to 2:25 pm
18µl
16µl
H'113 Not
H'113 Hind
H'113 NH
RT overnight until 6 pm Sun.
0.8% HX4 0.5 V/cm
Ag
M
Dye
W
Sep
Sample
W
Dye
Sun. Sep. 13th
Complete Not I VIII. 29
Take 10.6 µl (4 µg) R. H'113 VIII. 29
20 µl 10x RI buffer
167 µl ddw
2 µl (20u) Not 1 Lot 47
37° 6:55 pm to 10:30 pm → Cold
H'113 Not



(1) Conclusion
Not v. promising yields.



(2) Conclusion Not worth pursuing.

① On

① ~1hr
On 0.8% H×4 max

Dye
W
Pre
1
2
4
8
16
24
H'113 N/H
Ag MM
Acry MM
W
Dye

Mon. Sep. 14th

Progressive Hind III on VIII .29

Hind / Not — Hind … Hind

Take whole (200μl) ~4μg H'113 Not (4 units)

{8 ddW, 1 10×R1, 1 Hind3}

16μl ✓ to ✓ 1.6μl 10×PBS → Pre

+ ~ μl 1/10 Hind III Lot 50

1	2	4	8	16
3.5 to 3.5	35 to 3.5	35 to 3.5	35 to 3.5	35 to ~3.5 (24)

16 to ~3.5

80° for 10 min.

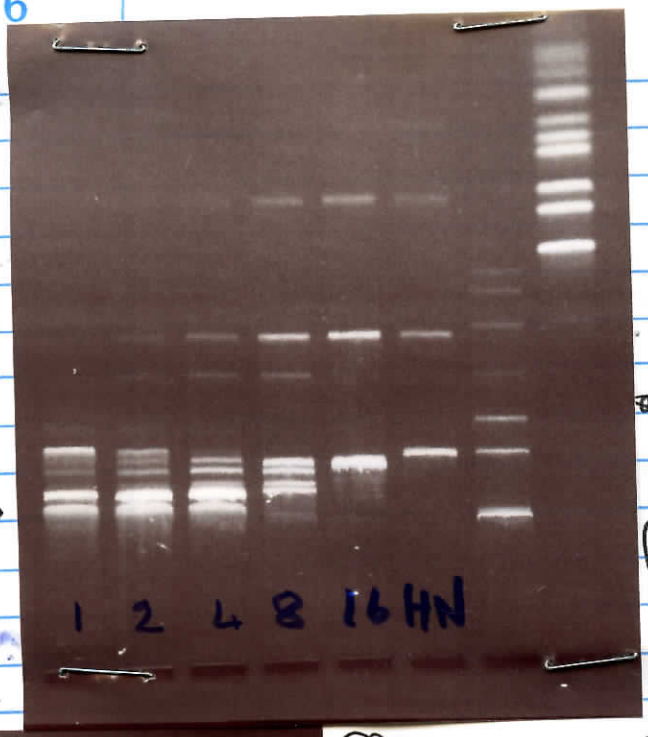
Ag On 7:15 pm 1.3 V/cm

0.8% H×4

Dye
W
5 slots ②④⑧ pool
Seap
λ HW #6
W
Dye

Pooled ② ④ & ⑧ on absence of 3.5 kb & presence of large. But yield will be low. Consider removing Hind at 3.5 kb — it should be in mouse intra

Also try single Hind followed by Not. Can use the single cut material for site change also.



?

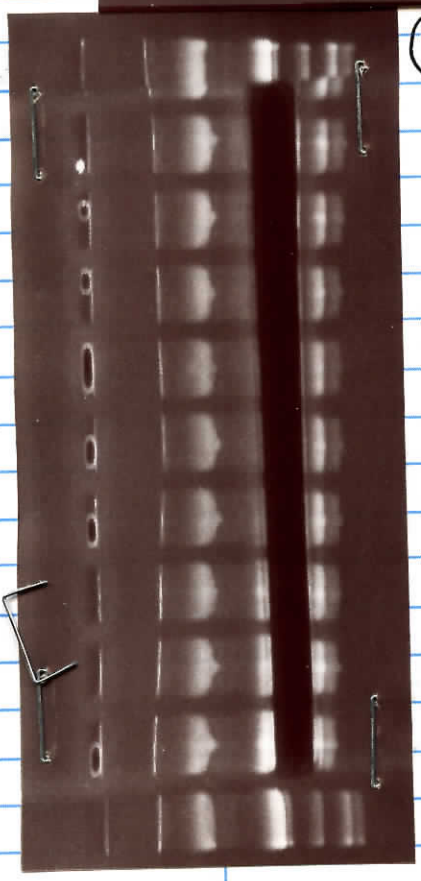
Conclusion ① ② ③ & ④

are good. ④ will have same

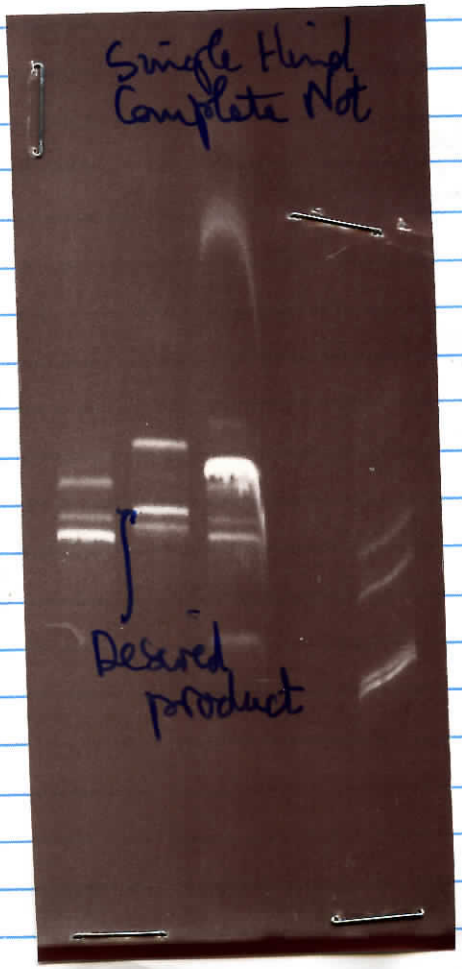
Hind Hind Not

But that is fine. Watch for nicked circ ~~[illegible]~~ or whatever ?) in ① ② 4 ③.

② Safe but left than perfect cut.



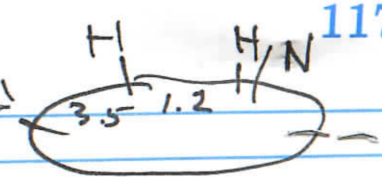
②



On 10:20
Off 11:15
0.8% HX4 mak

Dye
W
(1)
(2)
(4)
(8)
(16)
H'113 HN
AG
AC
W
Dye

Tues. Sep. 15th



Singlesite HindIII

Take ✓ 10.6 μl (4 μg) H'113 VIII.29
✓ 20 μl 10x R1 buffer
✓ 167 μl dd W
+2 μl 1/10 HindIII in R1 — lot 50 (20 u/μl)

[H'117 H]

Prewarmed

R1: ✓ 1 μl 10X R1, ✓ 8 μl dd W, → 1 μl HindIII lot 50

(1) (2) (4) (8) (16) min ✓ ✓ ✓ ✓

4 μl 10x PBS + 40 μl H'117 H ✓ 80-85° for 10 min

Pooled as ≥ 90 μl [H'117 ssH]

~10 μl + ~5 μl [H'117 mix] for check gel.

Sat pm On 2? Off 9:30am Sun
0.8% HX4
~1.2 V/cm

Sat Sep. 19th

(Cont'd H'123)

PRODUCT: pIRES-EGFP Vector

CATALOG #: 6064-1

AMOUNT: 20 µg

LOT NUMBER:
Specified on product label

STORAGE BUFFER:
10 mM Tris-HCl (pH 8.0)
1 mM EDTA (pH 8.0)

STORAGE CONDITIONS:
- Store plasmid at –20°C.
- Spin briefly to recover contents.
- Avoid repeated freeze/thaw cycles.

SHELF LIFE:
1 year from date of receipt under proper storage conditions

SHIPPING CONDITIONS:
Blue Ice (4°C)

FOR RESEARCH USE ONLY

DESCRIPTION:
A bicistronic vector designed for the simultaneous expression of the enhanced green fluorescent protein (EGFP) and a protein of interest from the same transcript in transfected mammalian cells. This vector contains an attenuated internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV) which permits both the gene of interest and the EGFP coding region to be translated from a single bicistronic mRNA.

CONCENTRATION: 500 ng/µl

PLASMID SIZE: 5.2 kb

CLONING SITES:
Cla I, *Eco*R V, *Not* I, *Eco*R I, *Bst*X I

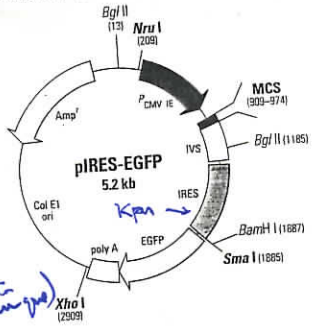
ANTIBIOTIC RESISTANCE:
Ampicillin (50 µg/ml; for propagation in *E. coli*)

PACKAGE CONTENTS:
- 1 tube of pIRES-EGFP Vector
- Vector Information Packet PT3157-5
- Living Colors™ User Manual (PT2040-1)

PT3157-5

pIRES-EGFP Vector Information Catalog #6064-1

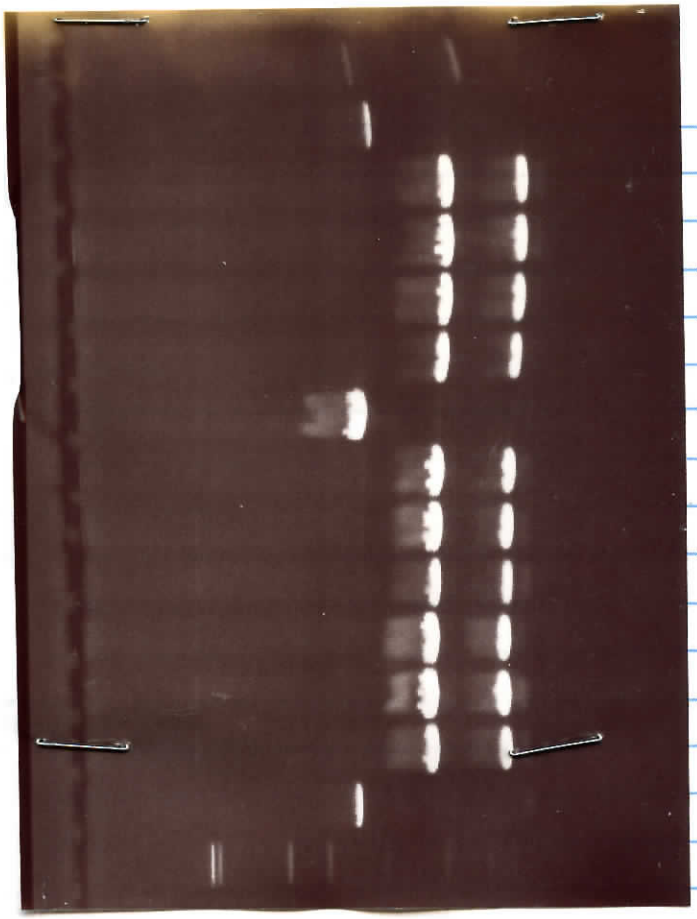
GenBank Accession #: Submission in progress.



900
CGAGCTCGGATCGATATCTGCGGCCGCGTCGACGGAATTCAGTGGATCCA
Cla I EcoR V* *Not I* *EcoR I* *BamH I*

950
CTAGTAACGGCCGCCAGTGTGCTGGAATTAATTCG
*Bst*X I

Restriction Map and Multiple Cloning Site (MCS) of pIRES-EGFP Vector. Unique restriction sites are in bold. The *Cla* I site (*) in the MCS is methylated in the DNA provided by CLONTECH. If you wish to digest the vector with this enzyme, you will need to transform the vector into a *dam*- host and make fresh DNA. The *Xho* I site can be used to linearize the vector

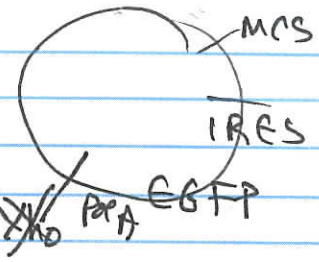


① Conclusion 1–4, 6–11 OK
10/12 5 & 12 unchanged.
Proceed

Processed all to KOAc & 12 to 0.001M EDTA.
Take ✓ 25µl 10x R4 salts
✓ 208µl ddW
✓ 7 µl Not-I Lot 47 (10u/µl)
37° 11:28am – 1:10pm
20µl + 1µl
Mins #1-9 #1-12

Sat. Sep. 19th

IRES-EGFP XhoI → NotI



H'119 Pre
H'119 Post

Take 133 µl dd w
15 µl 10x R1 buffer
(1µg) 2 µl pIRES-EGFP H'119 (from manufacturers)
~½ µl XhoI (10u) Lot 35 (20u/µl)

H'119 I-E XhoI

37° 1:15 pm to 2:35 pm

16µl → H'119 Pre

16µl → H'119 Post

Take (~0.3µg) 10µl H'119 I-E XhoI
+2.5µl 10 pg 95 ligase buffer
+1µl (~1µg) pg90 XhoI → NotI pHgo.
+9µl T4 ligase fdate Lot 67
15° 2:45 to 3:40 pm, RT 3:40 to 4:40 pm, 37° 4:40 to 5:05 pm

H'119 Ligs

5µl + 45µl 1xPBS

80° ⇐ 75° 3 min → ice

To cold

H'119 Heated Ligs

Transformation

H'35 DH5α
Heated Ligs

80µl
5µl

0° 5:16 pm to 6:05 pm

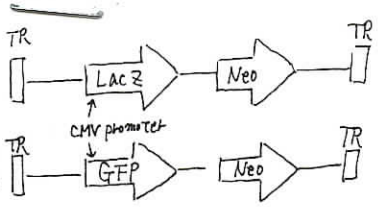
42° 90 sec

Many (18) (2) (0)

Plated 1xAmp NZY Rest, 20, 5, 20/16, 5/16

37° Sat 6:15 pm — o/n 8H Sm

Mini H'119 1–24
1xNZY 1xAmp 37° 6:45 pm Sm — 9:30 Mon (cont'd)



construct name

pABII

pTRUF5

Plate 10^6 ES (HM1) Cells in a 6-well plate (10^6 cells/plate)

↓ 1 day

Change the media

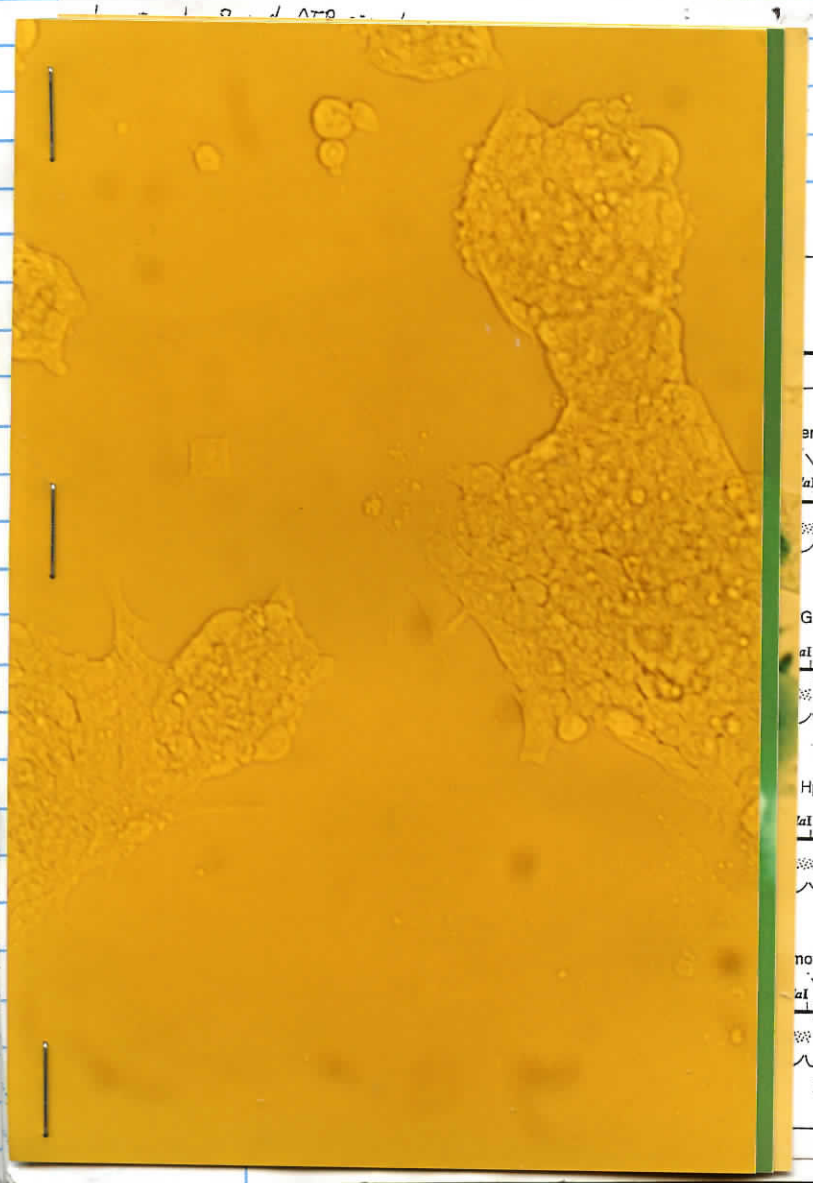
Put —μl Adenovirus

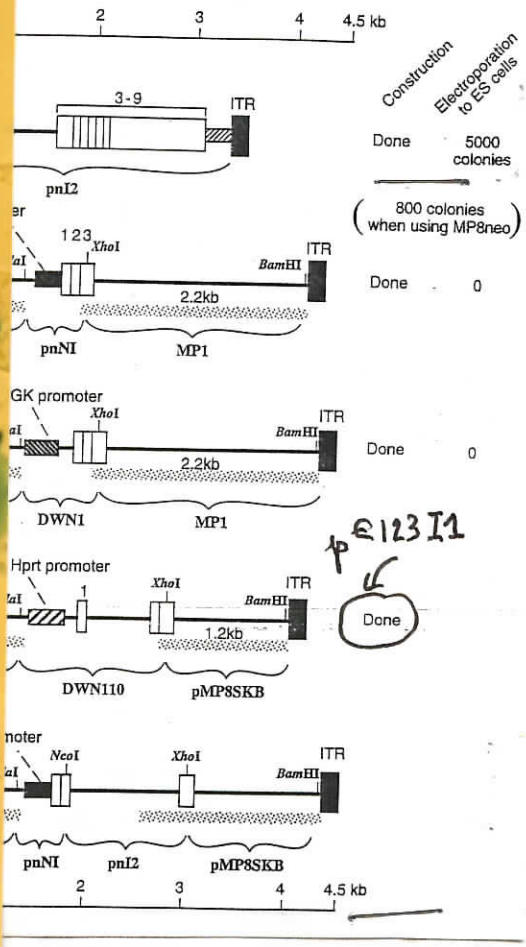
↓ 2 hours

Change the media

Put 10(~100)μl AAV-2, -3 or -4

↓ 1 day





Sat. Sep 19th

Update on transformation etc.

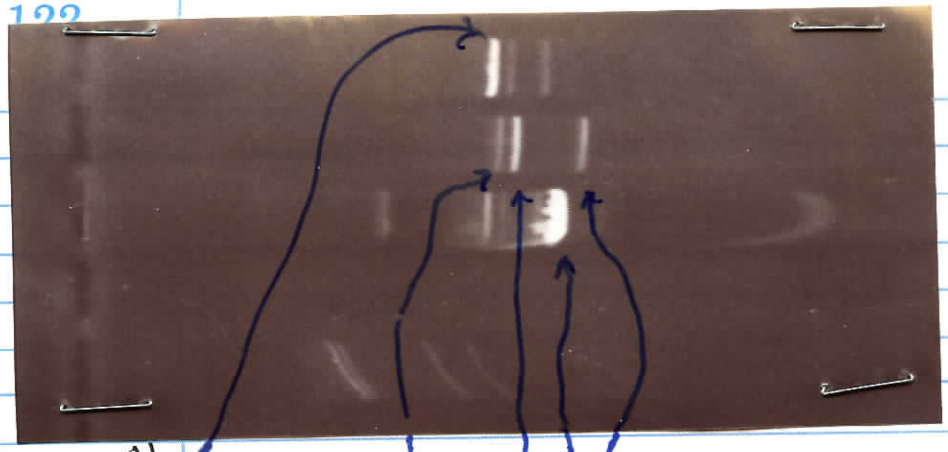
(1). Seigo's collaborator (Joe Patrinosity) finds that adenovirus pre-infection followed by AAV infection gives good infection of ES cells
- presumably still transient since at 1 day. without G418.
AAV2 is best.
Seigo will check stability & long term with G418 etc.

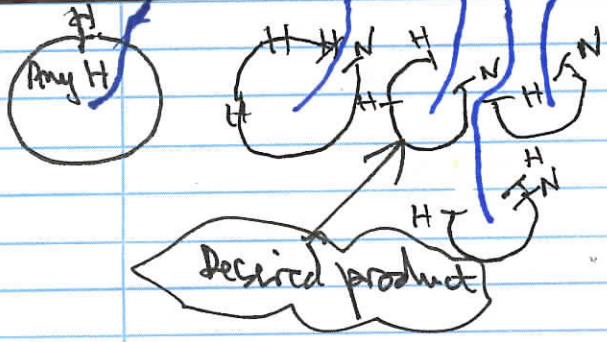
(2) pnI2 gave HAT^R colonies with ES cells.
∴ test when packaged into AAV2.

Nobuyo points out that we have been working with too many variables at once. Could have started with KH21 homology (already much reduced)
& change internal — but not really possible as pMPS correction sequence is > 4.5 kb.
pES123.I1 (rename pES123.I13) has essentially same length of homology as KH21 & uses exactly same 5' homology.

Later comment. The adenovirus induces double strand DNA formation ~~from~~ ss AAV but eventually all cells lyse!

Thurs. Sep. 24th — perhaps after AAV infection we can electroporate in an oligo primer to get ds DNA.





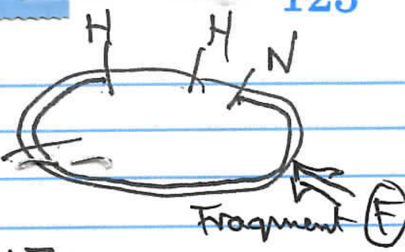
(1)

looks good - but MM went mad!! Yield will be ~ 1/3 to 1/2. Unlikely to improve by further purification.

① 1:18V
OK 5:265
0.8% ~ 2/3 max
H+4

Sun, Sep 20th

Hind - Not fragment H



Dye
W
VIII 29
H'123 Pre N
H'123 Post N
H'117 Mix
W
AG MM
W
Dye

Gel frag^t. recovery from H'117
is 100 µl of gel melt FOQ/ΦOH/CIAA
800 µl . . . .

whole into 80 µl
as H'123 H/N (1xR) salts

Back-up into 10 µl 0.001M EDTA
as H'123 VIII 29 Swift H
— can use for ~~Hind~~ if nec.

15 µl
H'123 N Pre

+1 µl (10 u) Not 1 lot 47 10u/µl

37°
12.05 pm to 1:05 pm

back to 37° until ? ~6pm

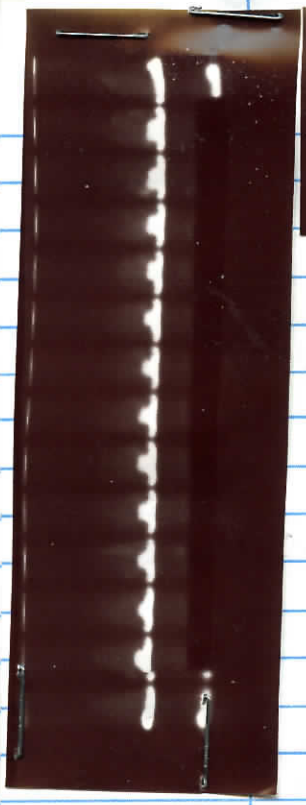
15 µl
H'123 Post N

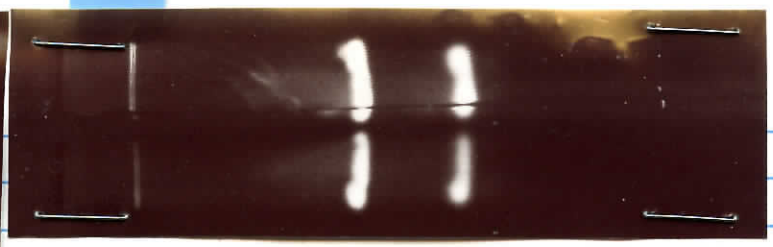
Photograph H'116 album ↗

CAUTION
Need to inactivate Not I before ligating.

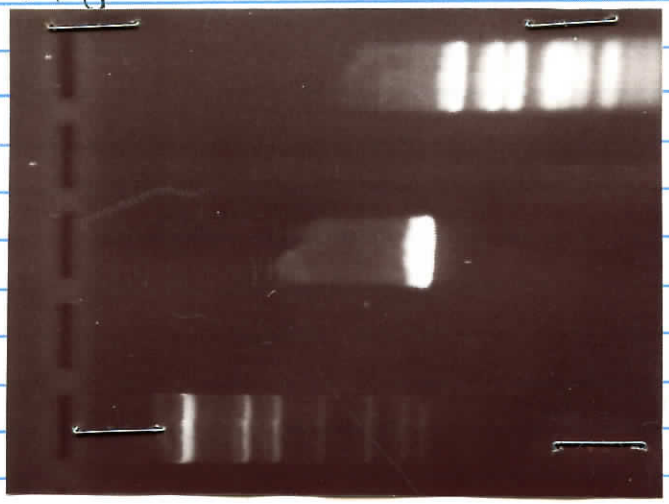
1x HOQ/ΦOH ✓
2x CIAA ✓✓

→ Renamed H'123 H/N Ⓕ

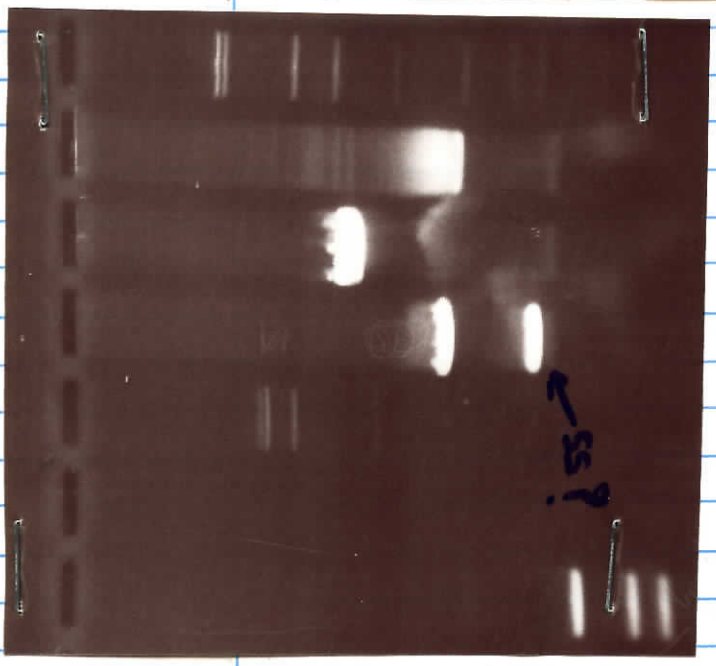




① Good cut.



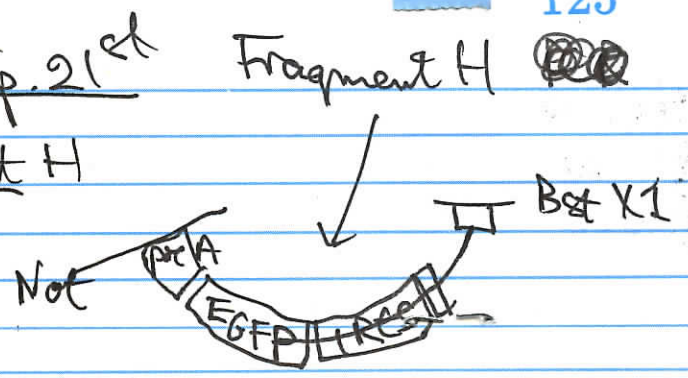
② Poor qd. Good fragment



③ Error in Labelling — but Bst X1 was true. Need to check H'125 (H) properly versus H'125 B/N (see H'127)

Mon. Sep. 21st

Prep. of fragment H



Take
- 30 µl 10×R1 buffer
- 255 µl ddw
- 15 µl H'119 mini #1
- 7.5 µl Bst X1 (75 u) Lot 4 (10u/µl) (5u/20 µl)

H'125 B/N

55° 4:30 pm to 5:15 pm

+ 7 µl Not I Lot 47 (70 u) 10u/µl

37° 5:20 pm to 6:30 pm

15µl Port B H'125

15µl Port BN H'125

H'125 (H)

Precipitated into 120µl as

Expected Eg 1.95 kb

H'125 Ptr

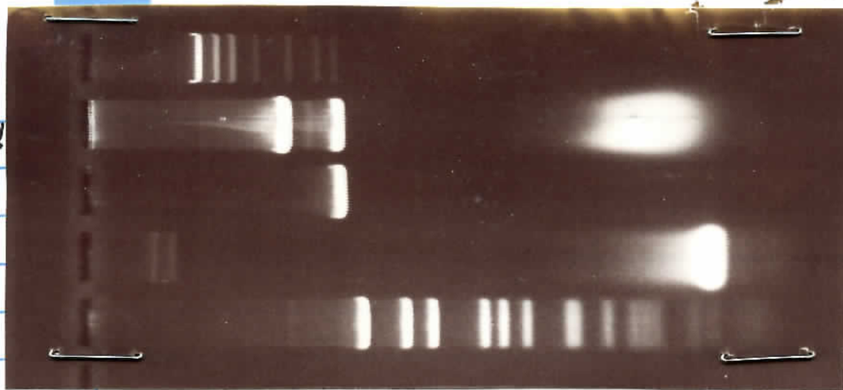
H'125 Port B Bst X1

H'125 Port Bst X1 / Not 1

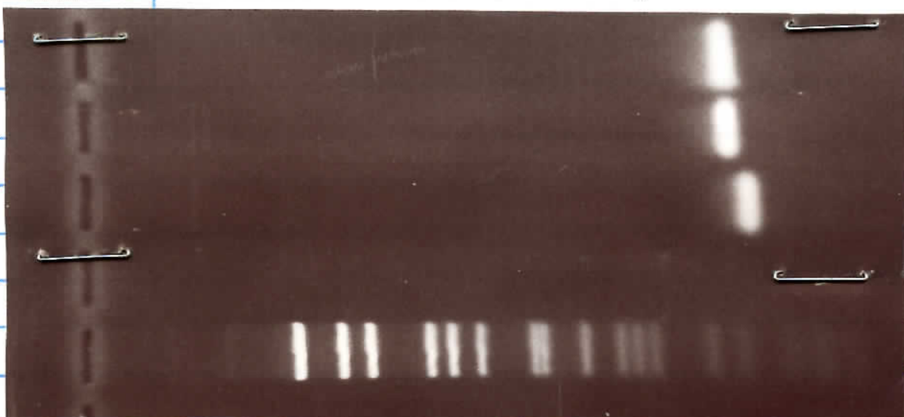
H'125 10 to 5 (F) ← Error, should have been (H) – it was (F)!

This was H'123 H/N

(2/3) B/N
(2/3) H
(4/3) PCR



① (H) is fine. PCR excellent yield & correct (115bp) size. Proceed.



② Can't certainly detect BstX I cut, so give an extra boost. See * below

③ Good cut.

Back up into 150 µl as

H'126 G#1

1.5% PFX7
~5V/cm
On 11:40 am
Off 12:20
③
Dye
W
~118bp
H'127 PCR B/H
W
Dye

* (Possible error) Biolabs says that a 2-base end on a BstX1 oligo yields only 25% cut in 2 hrs & 50% in 20 hrs. Could be a problem.

Wed. Sep 23rd

PCR for fragment (G) Fertility #1

n = 10

H'89	20 x PCR	✓	12.5 µl
	ddw	✓	190 µl
H'89	dNTP's	✓	1 µl
H'89	DMSO	✓	25 µl
H'110 Hp Exon 5	Red primer 1°	✓	5 µl
H'110 Hp Exon 5	Red primer 2°	✓	5 µl
H'110 Hp 4857	Blue primer	✓	5 µl
H'113 VIII.29	DNA template	✓	1 µl
H'89	Lot 42		Pfu polymerase ✓ 5 µl

H'127 PCR

into 5 tubes

≥90° ≥1 min
35 cycles 93° 1 min
60° 7 sec
60° 5 min

Start 11:40 am

Pool (~225 µl) + 23 µl 8M NH_4Ac ✓
650 µl EtOH ✓
Freeze. Cent. Dry ✓ back into 100 µl 0.001M EDTA
as H'127 PCR conc.

Wed. Sep 23rd HindIII & BstXI ends to PCR frag G.

Take 30 µl H'127 PCR conc
30 µl 10 x RI buffer
240 µl ddw
3 µl BstXI lot 4 (30u) (10u/µl)

H'127 PCR B/H

55° 5:50 pm to 6:50 pm

1.5 µl HindIII lot 50 (30u)

Pre H'127

2+18 of H'127 conc

10:35 am 55° + 3 µl BstXI

H'127 Post B

A.T. alternate

37° 6:55 pm

H'127 Post B/H

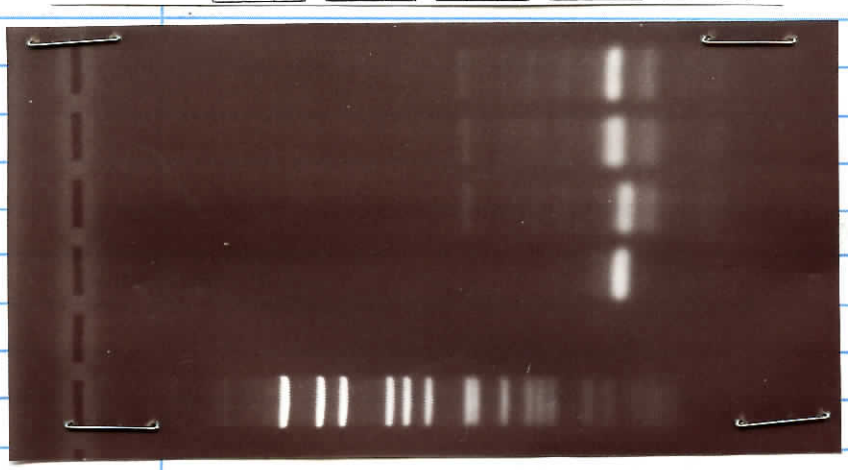
cold overnite

11:35 am

H'127 Post B/H

Oligo # 20161 5' Oligo Name: H'129 HpEx5 Red 3° 3'
Date made: ____
LENGTH: 23
%G+C: 41%

CAA	GTC	TCC	AGC	ACA	
CTG	GCC	AG			



① Product is not good. Repeat PCR with DNA template → Blue, Red 1° → Red 3° primers. Meanwhile, work on H'131 product.

Oligo # 20198 5' Oligo Name: H'128 HpEx5 Red1° 3'
Date made: ____
LENGTH: 37
%G+C: 54%
plate-purify

CAA	GTC	TCC	AGC	ACA
CTG	GCC	AGC	CTT	GCA
TTA	GTT	C		

Oligo # ____ 5' Oligo Name: _____ 3'
Date made: ____

Mar. Sep 28th

Shared 35 cycles

20x Pfu 8 min ext.	12.5
ddw	190.5
dNTPs	1
DMSO	25
4857 Blue	5
H'128 HpEx5 Red	5
H'129 HpEx5 Red	5
VIII 29 Template	1
Pfu	5

Pearish start at 4:40pm
Recovered in 100µl as H'128 PCR conc

16µl PRE H'129

On 9:22

Total
+ 2
+ 6
Freeze
cuts
Take
+2
+
5

(F)

Fragment G2

9:22 am
Off 9:52 pm

Hx 1
2/9 MAX

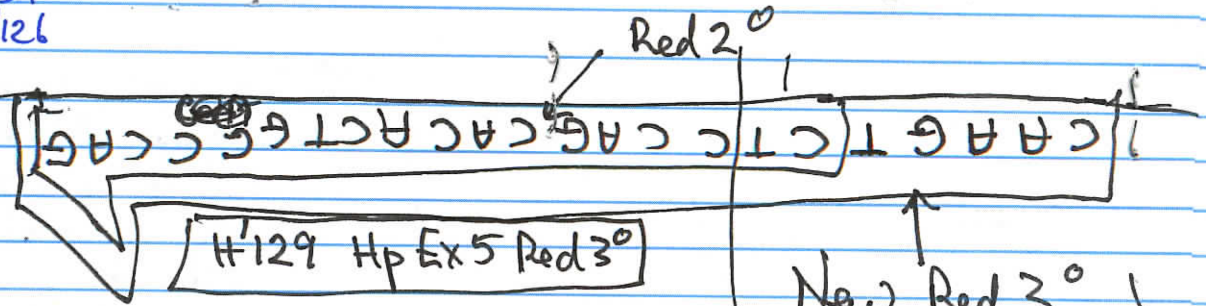
Thurs. Sep 24th

Back-Up Correction of PCR fragment G

Reamplify H'127 PCR conc. with old primer H'110 Hp 4857 Blue plus a new 3° Red primer with more ~~[illegible]~~ extension for Bst X1

(H' 2/9)

- 1) Dye
- W
- Pre
- Post B
- Post B/H
- G#1 H'126
- W
- ACRMM
- W
- Dye



M.P. will be 76°, but should be OK

Not well designed - need to make longer 1° also - see

Fri - Sep 25th

G fragment PCR (n=10)

				Repeat
H'89	20×PCR	12.5 µl	✓	✓
	ddw	195.5 µl	✓	190.5
H'89	dNTPs	1 µl	✓	✓
H'89	DMSO	25 µl	✓	✓
H'110	Hp 4857 Blue primer	5 µl	✓	✓
H'129	Hp Ex 5 Red 3° primer	5 µl	✓	H'113 VIII
1/10 H'127	PCR conc template	1 µl	✓	1 µl
(H'89 +) H'129	Pfu polymerase lot 37	5 µl	✓	5 µl new

Repeat: H'110 Hp Ex 5 1° Red

into 5 tubes ≥ 90° ≥ 1 min
35 cycles 93° 1 min
Start 10:05 am 40° 7 sec
60° 8 min. (sharing)

Total vol ~ 250 µl
+ 25 µl 8M NH_4Ac
+ 688 µl EtOH
Freeze. Cent. Dry. Back into 100 µl 0.001M EDTA ✓
as H'129 PCR conc

Take 30 µl H'129 PCR conc
+ 30 µl 10×R1 (H'129 B/H)
+ 240 µl ddw
+ 3 µl Bst X1 lot 4 (30u)
55° 5:30 to 6:30 pm

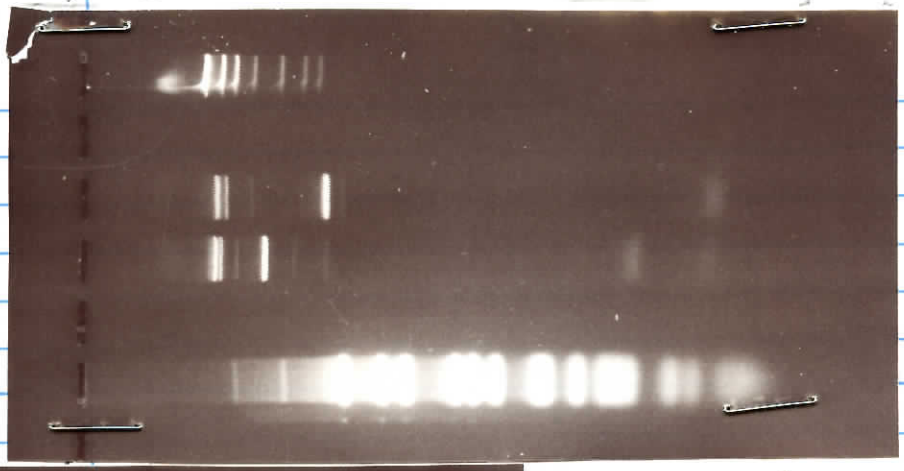
16 µl PRE H'129

30 µl POST B H'129

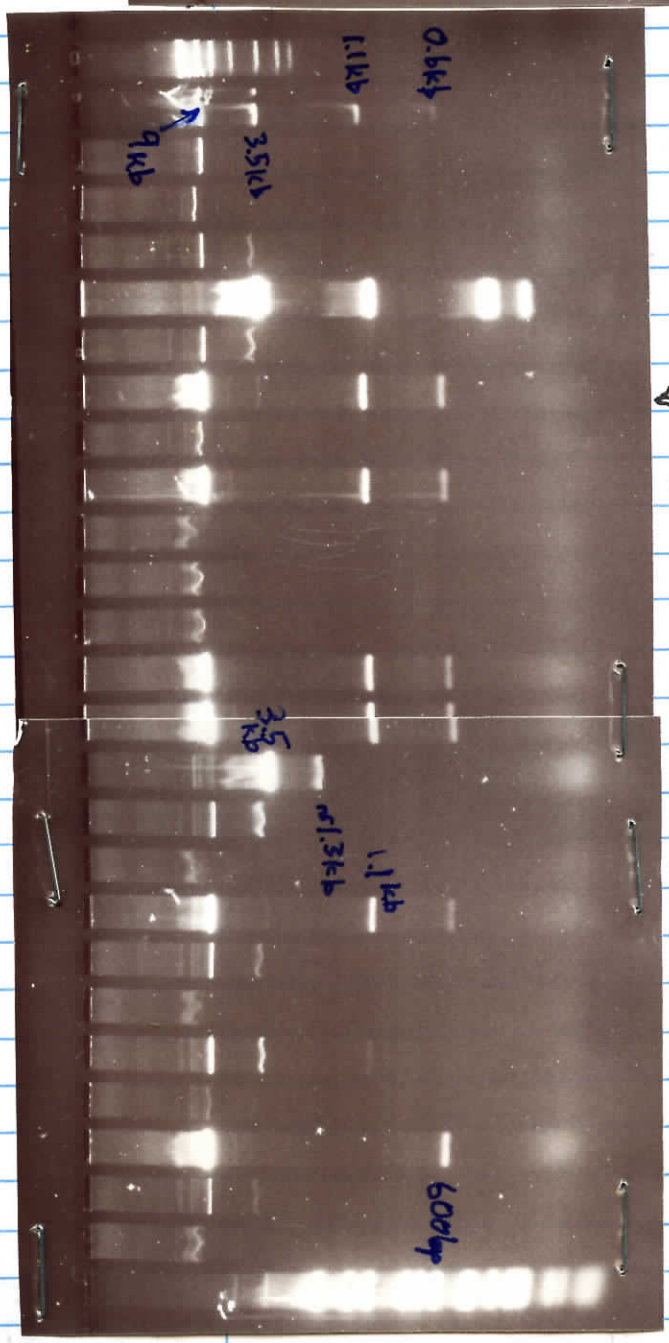
+ 1.5 µl Hind III lot 50 (30u)

37° 6:40 pm to 9.05 pm → to cold, 16 µl Post H'129 B/H

Recovered gel as 100 µl H'129.2 G conc



① Conclusion
Good ligation
- but a lot
of ferment!
Proceed — ?
work back up.
Preparations are
good.



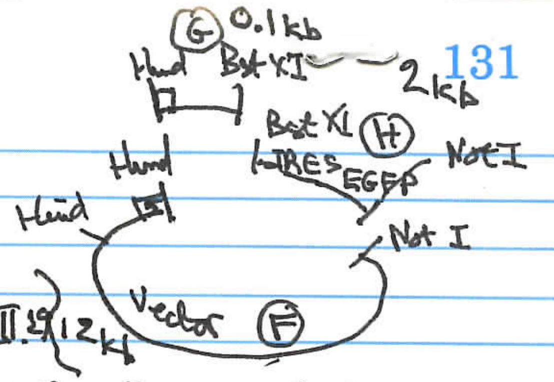
② Conclusion Almost
certainly the Bst X1
cut is the problem,
since no clones have
the H-B-N 2.1 kb
fragment. Try again
to get Bst X1 to
work. Order
new longer Red !°

← Parent - 3.5
← -3.5
← -3.5
← -3.5
← Parent - 1.1 - 0.6
← -3.5
← -1.1 -0.6
← ? Parent or -0.6
← -3.5 -1.1

Diagnostics :-
VIII, 29 will
give: 0.6 kb H/B
1.1 kb H/B
3.5 kb H/B
9 kb H/B
Desired will give
H~~S~~N 2.1 kb H/N
3.5 kb H/B
9 kb H/B

① 1.5% HX1 10V/cm
On 11:32am Off 11am

Thurs. Sep 24th



w/ AgMM Assembly (1st try)

Dye W — Total size will be slightly larger than VIII. ~12kb

Pre H'131 — ✓ Take 15 μl H'123 H/N (F) (~45% of total)
Post H'131 — ✓ 7.5 μl H'125 (H)
W — ✓ 7.5 μl H' 126 (G#1)
Acr. MM — ✓ 3 μl 8M NH_4Ac
W — ✓ 83 μl EtOH
Dye — Freeze. Cent. Dry. Back up

On 3:... Off ... 3.5% HX1 1.56 RNAse MIX — ✓ into 15 μl 1x p95 as
✓ + 1.5 μl T4 ligase Dilute Lot 26 — H'131 LIG^N

② Dye AG MM H'113 N/H H'131 mini 1 H/N 2 ... 23 24 — 3 μl + 12 Pre H'131 — H'131 Hind/Not minis

15° 3:53 pm to 4:55 pm
RT 4:55 to 5:27 pm
37° 5:27 pm to 6:15 pm — to cold

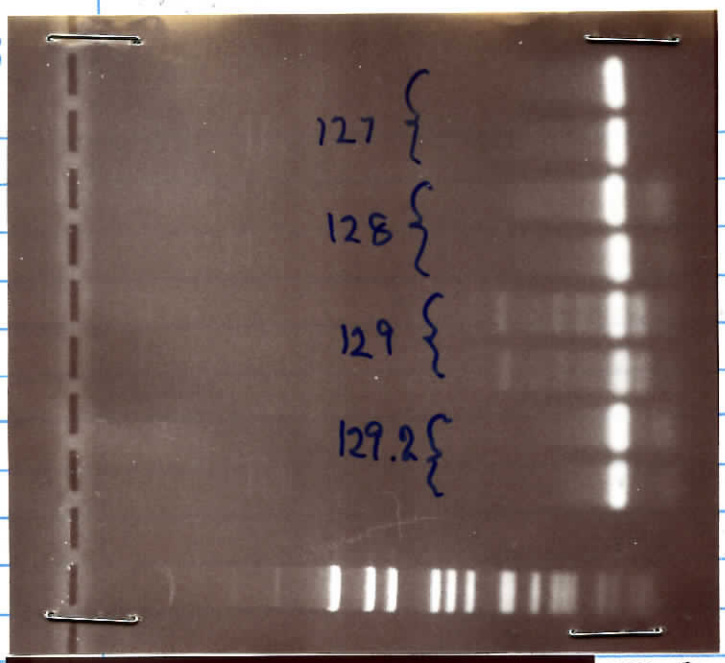
3 μl + 12 POST H'131

Acry M W Dye

Transformation

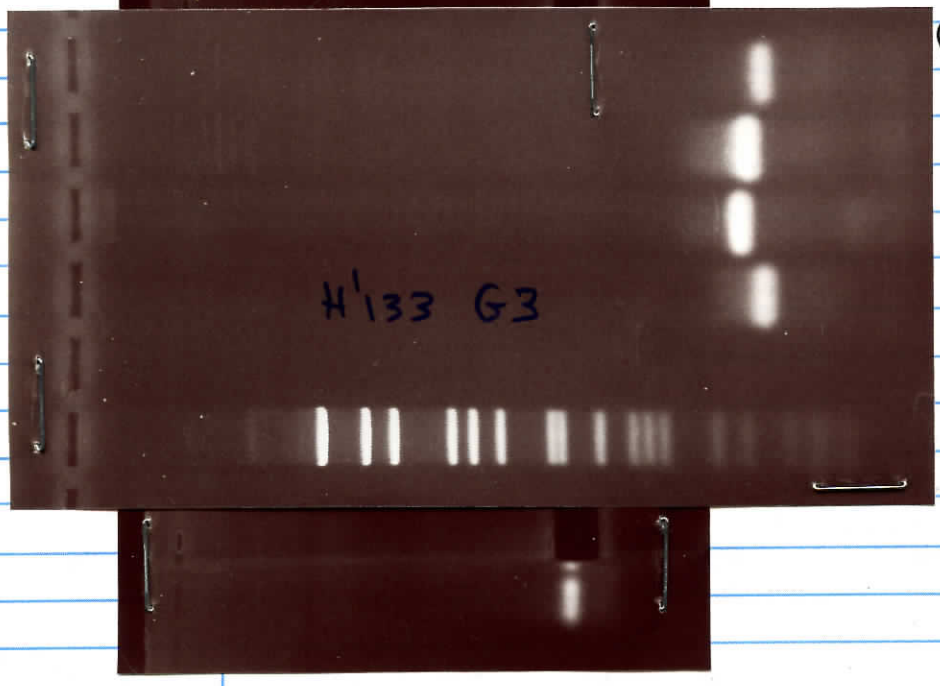
H'35 DH5α 80 μl ✓
H'131 lig^N → 3 μl ✓
0° 6:15 pm to 7 pm — ~ 9/10 ratio
42° 90 sec — 2L/8S — 1L/4S ← Small & large (normal) colonies
Plated R 20, 5, 20/16, 5/16
1X Amp NZY 37° 7:15 pm

Picked as good representation of sizes as poss.
1K Amp NZY 37° good shake at 3:10 pm Fri — 10am Sat
Processed to minis — simplest diagnostic is (Hind III digest & 0.5% gel / Not I)
Take ✓ 52 μl 10x Ad salts ✓ 6.5 μl (130) Hind III Lot 50 — 1 μl each mini
50/sample 450 μl ddw — ✓ 13 μl Not I Lot 47 (130U) — 37° 2:45 pm to 2:50 pm



① Conclusion: Clear result with 128 — Bst X I shortened it. Proceed.

② Conclusion Hind cut + gel cut are OK.



③ Yield fine. Cuts confirmed.

Tues. Sep. 29th
~~Sat. Sep. 26th~~

H PCR Bst X1

Bst X1 tests on PCR products (prior to Hind III)

Oct 7 1:58am Off 9:28am
Dye 9 HX1 1.5% Max

Four ~~three~~ PCR products ~~are~~ available:

(1)
Dye
W
Pre 127
Bst B H'127
Pre 128
Post B H'128
Pre 129
Post H'129 B
Pre 129.2
Post B H'129.2
W
Marker
W
Dye

✓ H' 127 PCR conc.
✓ H' 129 PCR conc.
✓ H' 129.2 conc.
✓ H' 128 PCR conc.

H'127, 9, 9.2, 8 B(st X1)

Take 30 μl of each + 270 μl
{10x R1 buffer 120 μl
ddw 1080 μl
(Lot 4 100 u/μl)

and 30 μl of each as
PRE 127
PRE 129
PRE 129.2
PRE 128

+ 3 μl Bst X1 to each
→ 55° from ~11am to ~1pm
Frozen
30 μl of each as POST

Wed. Oct 7th
← Gel on PCR

H PCR Bst X1

Further digestion of H'128

Take whole of H'128 B(st X1) digest
+ 1.5 μl (30 u) Hind III Lot 50
37° 10:15am to 12:35pm

H'133 128 BH

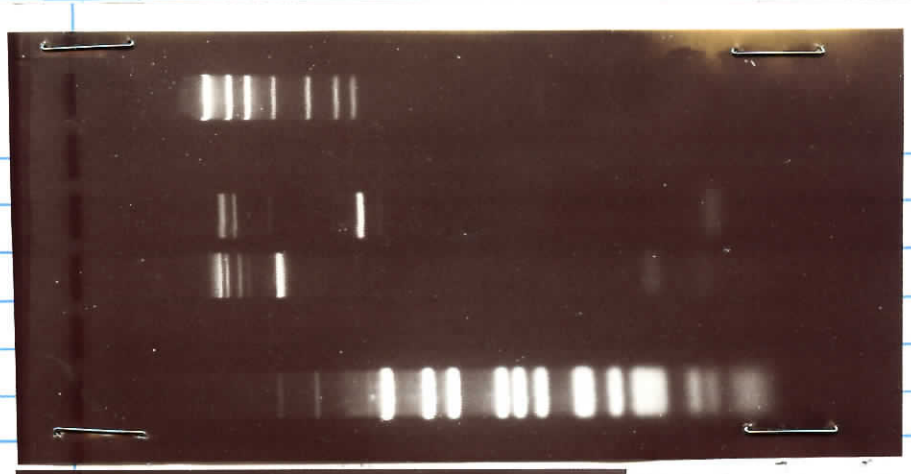
30 μl H'133 128 Pre H

Recovered into ~~Dye~~ 150 μl as H'133 G3

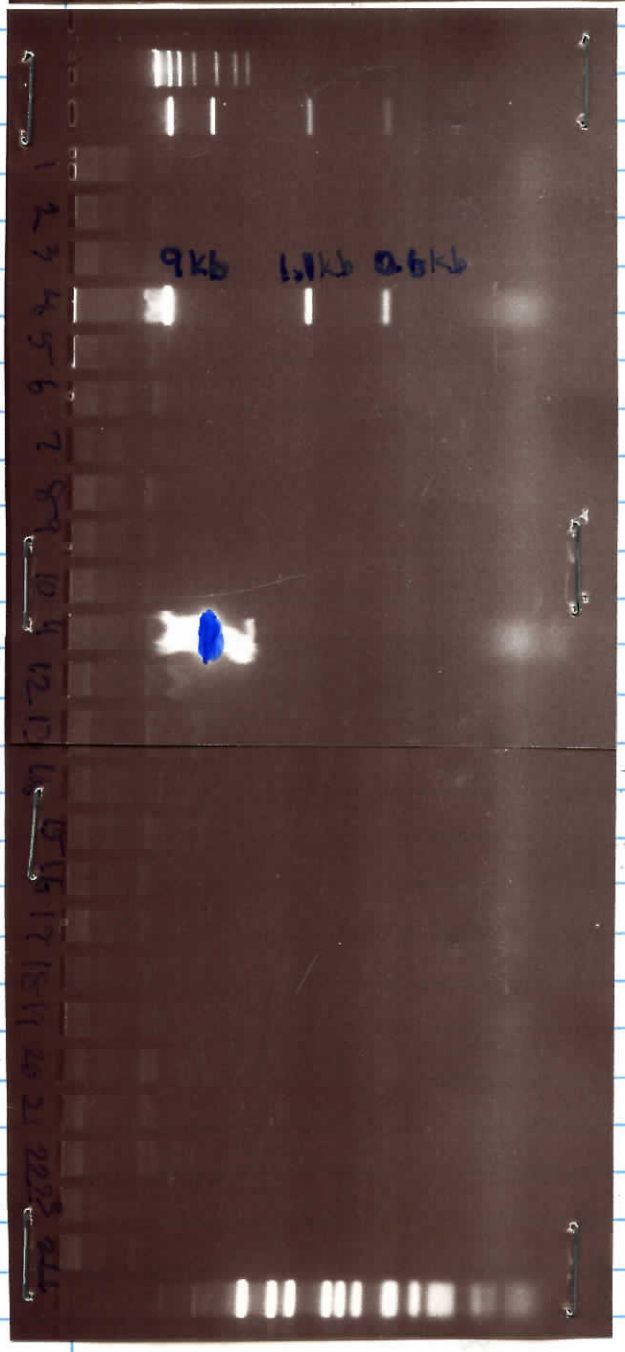
On 12:45pm Off 2:20pm
1 slot 128 Pre H'133
Dye
W
~ slots
W
Dye
Ag
1.5% HX1
~5 V/cm
SeaPlaque

(3)
W H'133 128 BH
H'133 128 Pre H
[illegible] Pre 128
H'133 G3
W
Marker
W
Dye

2% HX1 Max
On 4:55pm Off 5:25pm



① Conclusion
Migration looks good - marginally better than H'130 - but proceed



~3.5

② Most surprising - all the tiny colonies gave nothing - but clearly #4 (almost certainly the colony w/ normal size) grew well ~~[illegible]~~ decided to pick another 2# from H'135 but of normal size. See H'137.

① On 12:20pm
Off 12:50pm Hx1
1.5% 10V/cm

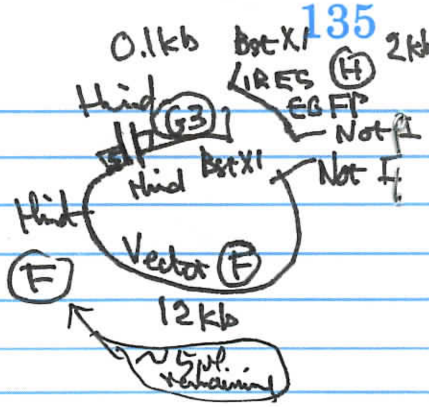
Dye
Water
Ag MM
W
3+12 Pre H'135
3+12 Post H'135
W
Acry MM
W
Dye

② On 1:56pm
Off 2:30pm Hx1 1.5%
+Ag 10V/cm

Dye
Ag MM
H113 H/N
1 } H'135
2 } Minis
! } H/N
23
24
Acry MM
W
Dye

Wed. Oct 4th

Assembly repeat



Take ✓ 15µl #'123 H/N (F)
✓ 7.5µl H'125 (H)
✓ 7.5µl H'133 (G3)
✓ 3µl 8M NH4Ac
✓ 83µl EtOH
Freeze. Cent. Asp.
Back into 15µl 1x p95 as [H'135 LIGN]
+1.5µl T4 ligase
Invitrogen Lot 26

3µl + 12µl [Pre H'135] To cold room 6:30pm
3µl + 12µl [Post H'135]

8:30am 10.35 RT → 11:38 am → 37° → 12:08 pm
15° / 16°
To cold

Transformation

H'35 DH5α	80µl
H'135 Lig^n	3µl
0° 1:40pm to	2:25pm
90 sec. 42°	✓18 small / 21 ; 3/4 small
Plate & Rest	20, 5, 20/16, 5/16 37° 2:35pm to
1x Amp	NZY

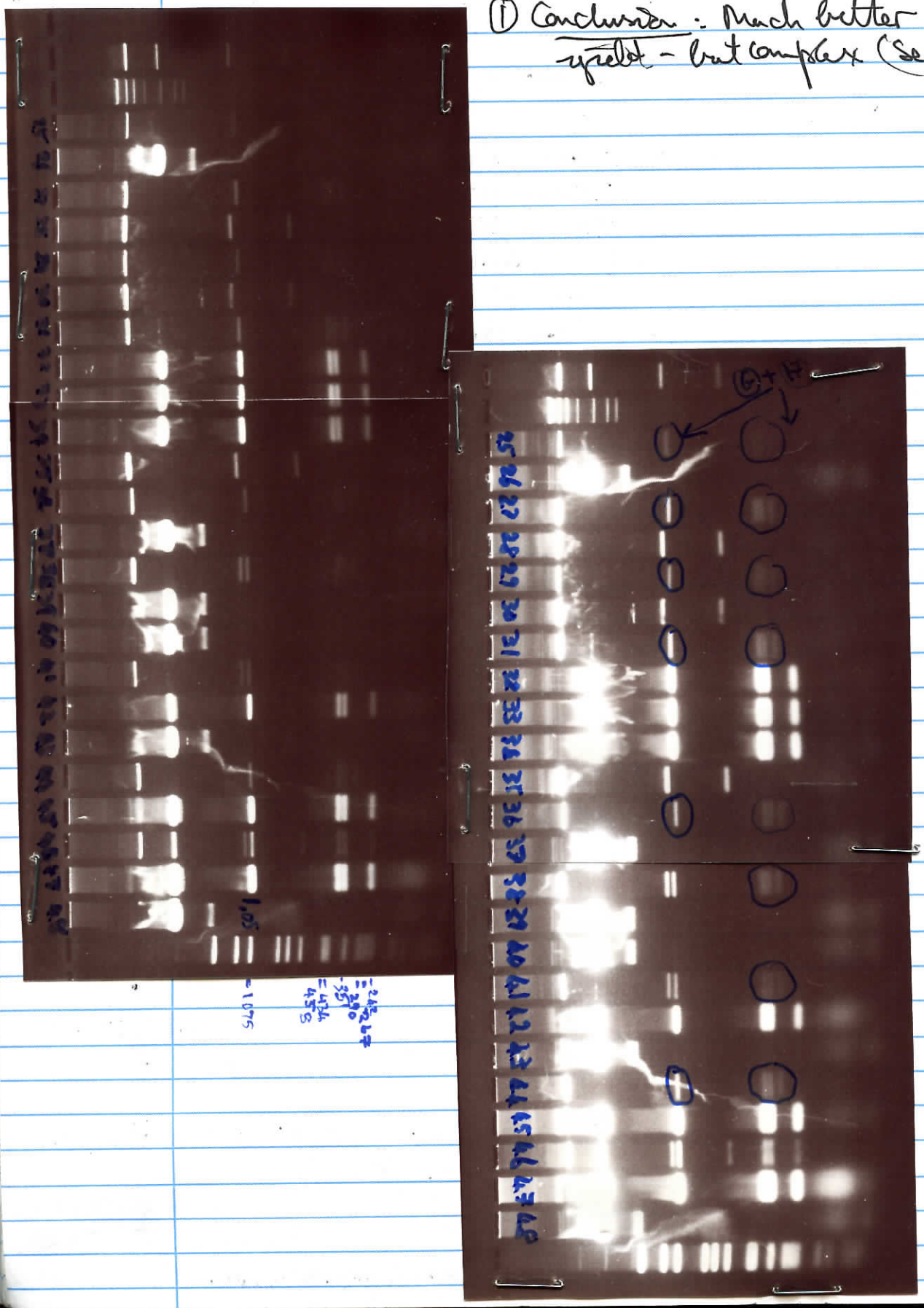
1:30pm tue
→ to cold.

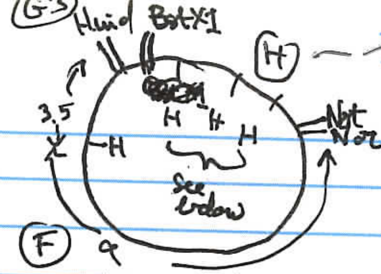
Sun. Oct 11th

Picked 24 to 1x Amp NZY minis H'135 37° @ 2:25pm
plus 4 extra from 20/16 to replica only

Proceeded to minis: Take / 104µl 10x R1 salts + 13µl (260u) Hind III Lot 50 + 26µl (260u) Not I Lot 48
also 40µl / +900µl ddw / +2µl mini (≡5u / 1µl sample)
37° 12:26 to 1:35pm (Lost some of #2 - Solution incomplete on several)

① Conclusion: much better yield - but complex (see ↓)





On 1:47pm
Off 2:26pm
1.5 μg H x 1
RNAse 2nd 11am
MAX

Mon. Oct. 12th

More minis

① Bye — Ag MM
H' 113 H/N
H' 137 #25 mini H/N
#.2 Picked H'135 minis #25 – 48 but with ~normal sized colonies only. To 1x NZY Amp 37° 6pm
No "tinies". Proceeded to 50 μl minis & Hind + Not as H'135 to 37° 11:24am — 1:24pm
↑ 2 minutes earlier & later start!

All grew on replica ~normal

25 – 48
47 } H'137 H/N
48 }
Ag. MM
W
Bye

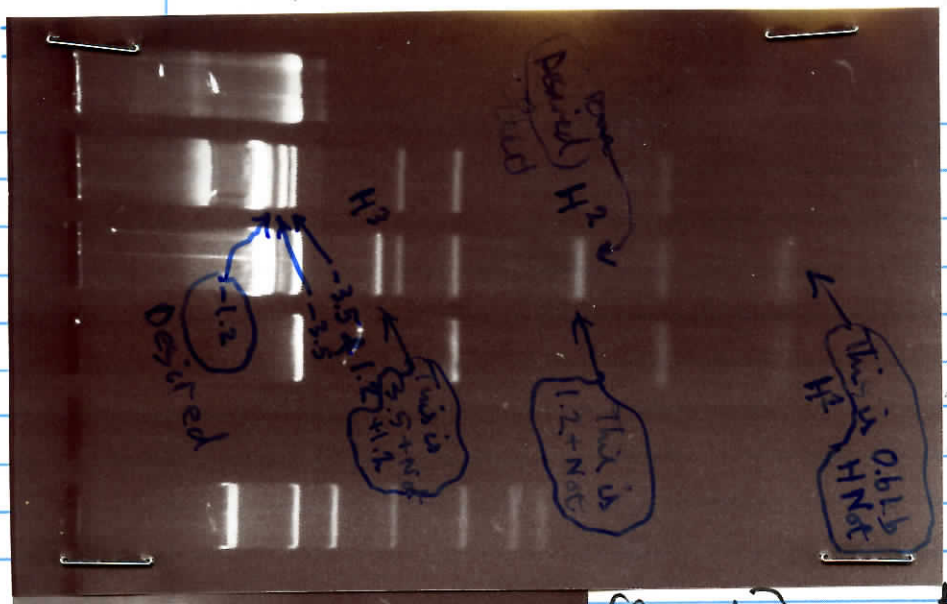
Conclusion: No real improvement
— can't find the desired product.
? Go back to making a single Hind III
— or else there's a fundamental error

Yes, there is an error — in thinking: there are 3 Hind III sites in fragment (H) !!

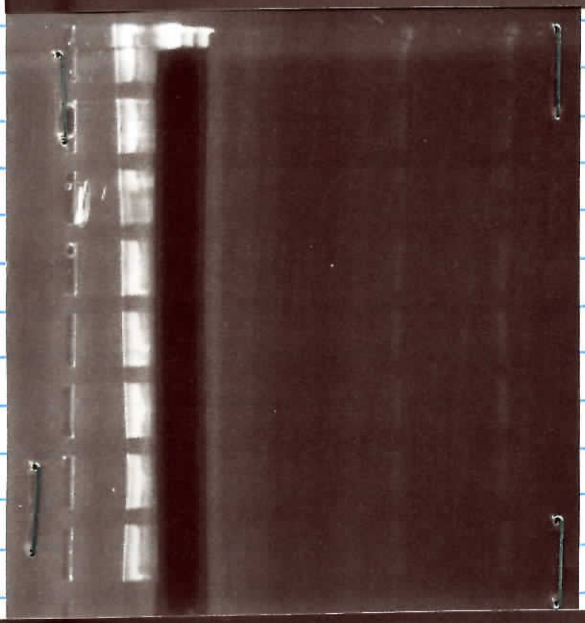
Need to go back & look at old & new colonies — the triplet in 32, 33, 34, 42, 45, 46 etc. is possible — but the one in 7 & the like needs study.
New pattern sh'd be (9 kb)(3.5 kb) 300bp, 360bp, 350bp, 1050bp —
(F) (G + H)

(45) may be an impure example of desired — but it looks like a mixture of (47) + (35) on better examination.
Plate more of H135 lig.^n & screen normal colonies.
On more careful inspection it is clear that the problem is absence of the Hind III 3.5 kb band of the vector. [illegible]
[illegible] So the problem is fragment (F) [illegible] altho' it looks ok.

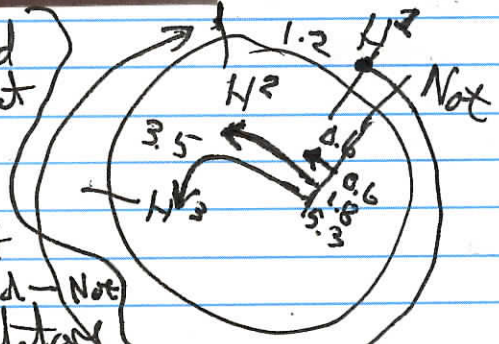
Make it again!
Meanwhile picked (49) — (72) 1x AmpNZY 37° 6pm — Th. Oct 15th



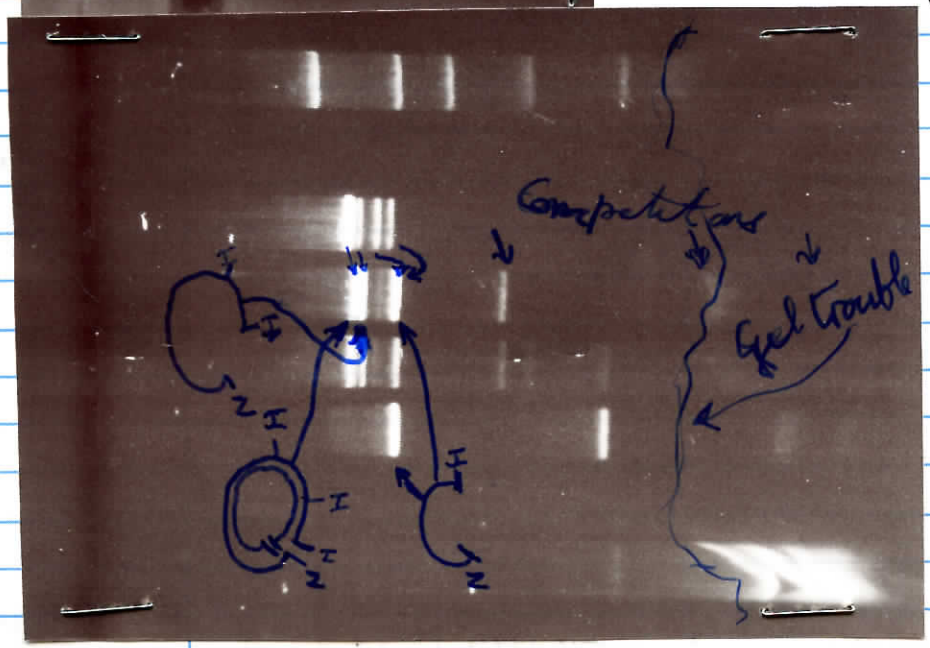
① Conclusion
Equivalent to 4 min. on previous test - as expected. But it looks as if the desired Plind site is a poor cutter.



② Good cut, but there will be a lot of third — Not completely after Not I.
Probably best to purify again & accept losses for purity.

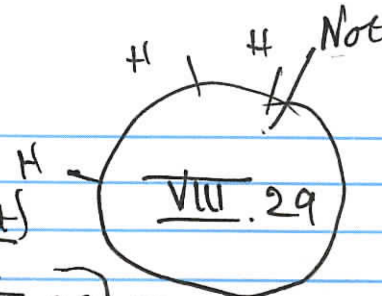


③ Conclusion: It's clear that a second purification is worth the losses. Proceed.



On 2:32pm
Off 3:39pm
0.8% Hx4
Marx

Thurs. Oct 15th



① Dye
W
H'139 Pre
H'139 VIII H(Post H)
H'139 Single H Not I
H'113 H/N
W
Ag MM
W
Dye

VIII.29 single HindIII (repeat)
± NotI

✓ Take 10.6 μl (4 μg) H'113 VIII.29
✓ + 20 μl 10x RI buffer
✓ + 167 μl ddw
✓ Prewarm to 37°
✓ + 2 μl 1/10 HindIII in R1 — ✓ 1 μl 10x R1, ✓ 8 μl ddw, 1 μl HindIII Lot 5
✓ 2 min. 37° & then 80-85° for 10 min. → Ice

H'139 VIII H

✓ 30 μl → H'139 PRE

✓ 30 μl
✓ + ½ μl (5u) NotI Lot 47 (10u/μl)
37° 1:23 to 2:23pm

H'139 Single H Not I

can check which is most likely for purification.

② Dye
W
8 slots
0.8% Hx4
Seap & Ag
On ~5pm off ~10am
1.2 V/cm

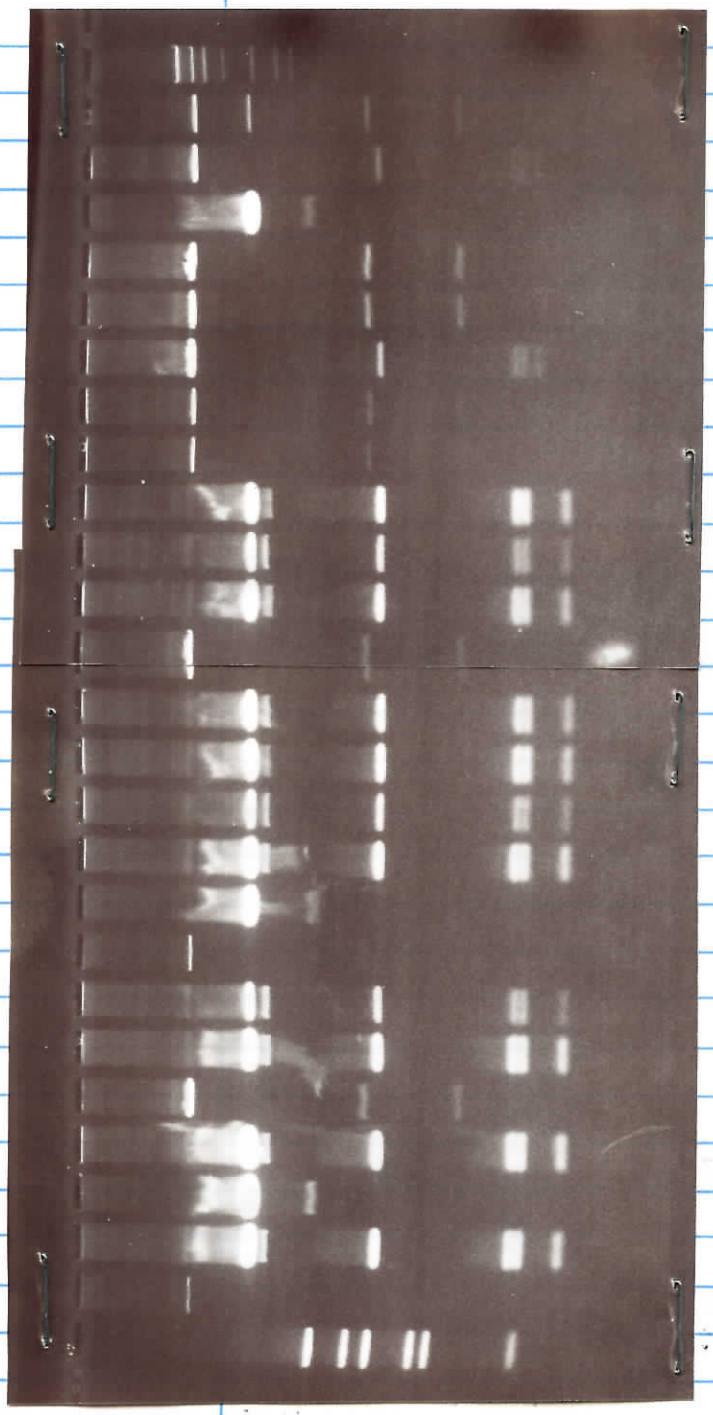
③ W
Dye
On 1:43pm
Off 2:43pm
Dye
W
ACR MM
W
H'139 VIII HN Pre
H'139 VIII HN Post
H'139 single H Not I 4+12
H'113 NH
AG MM
W
Dye

Recovered into 30 μl 0.001M EDTA as H'139 VIII Hss

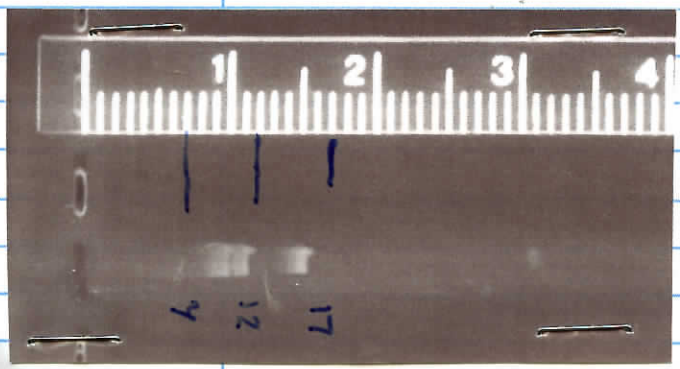
+ 8 μl 10x R1 salts
+ 1 μl (10u) NotI Lot 48 ✓
to 37° 12:31pm to 1:31pm & back to r.t.

✓ 15 μl → H'139 VIII HN Pre

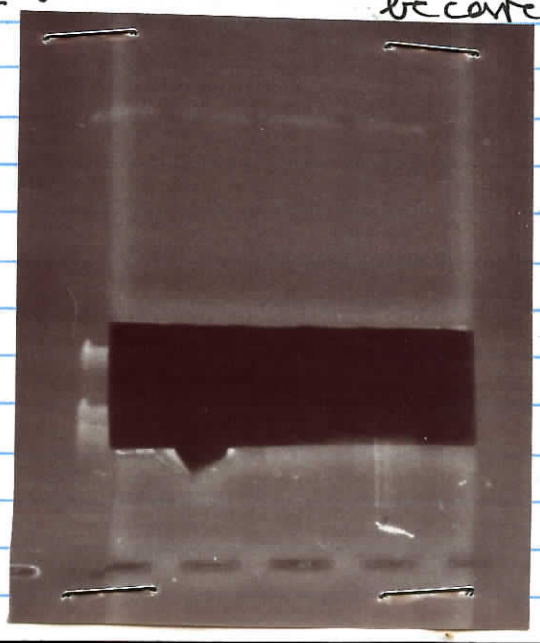
H'139 VIII HN



① Conclusion: The 3.5 kb band is conspicuously absent!



② Cut into two fractions F(F) & S(F) expecting S to be correct.



(F) S

① 3:53 On ~4.30 Off 9H×1 RNHisd 1.5 bio 8 V/cm

Fri. Oct 16th

More H'135 minis & reprep of fragment (F)

Dye
AgMM
H'113NH ✓ Take 52 μl VIAR1
49 ✓ 450 μl ddW
50 H'141 minis H+N ✓ 6.5 μl Hind III lot 50 (5U/sample)
! 13 μl Not I lot 48 (5U/sample)
71 ✓ 20 μl aliquots + 1 μl of minis (49) — (72) H'135
72 37° 1:07pm to 3:30pm
AgMM
W
Dye

② 0.8% H×4 Ag Fri On 6:30 pm Sat Off ~12 noon
1.5 V/cm

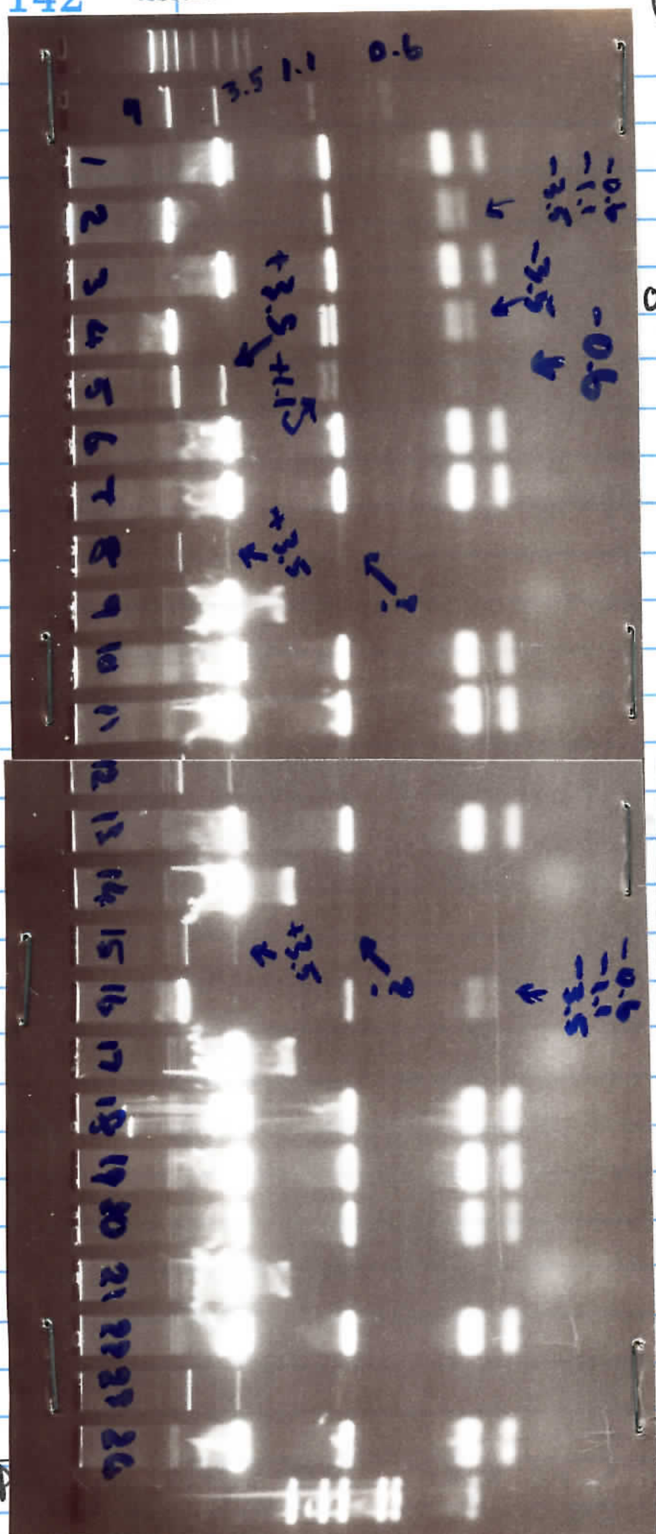
Dye Seap
W

4 slots only (!) of H'139 VIII HN

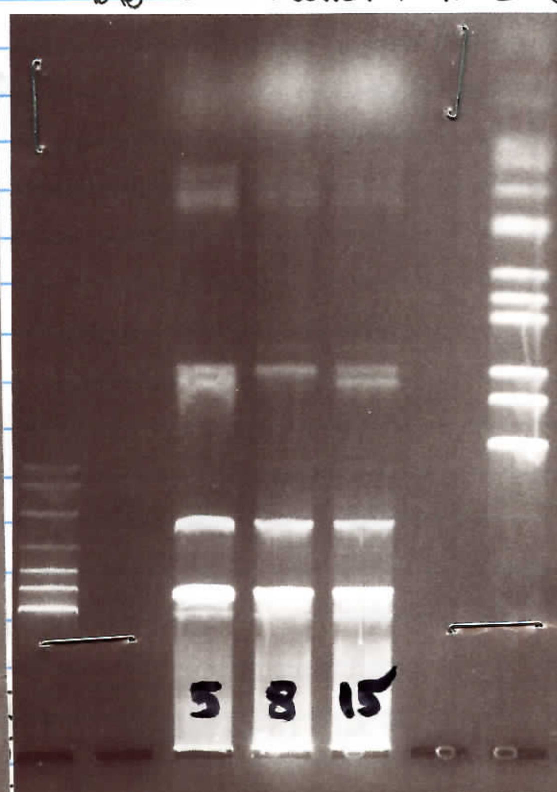
W Recovered into 30 μl as H'149 S(F)
Dye

and H'149 F(F)

Proceed with S(F) first!



① conclusion: Pick more — & more medium small. The fragment (G3+H) is the one vector that is minus 0.6 kb H/H & one that is minus 3.5 kb H/H but not one that is minus 0.6+1.1 kb H/H & +1.05+301+350+360 and one is present that is minus 0.6 minus 1.1 and minus 3.5.
It may be possible to derive the desired product from colony ⑤.
Colonies ⑧ & ⑮ need testing at higher conc.
Meanwhile pick another 24.
Did so — minis H'143 ㉕–㊽ 37° 6pm Mon → Tues am
Processed to 50µl



µl NotI Lot48

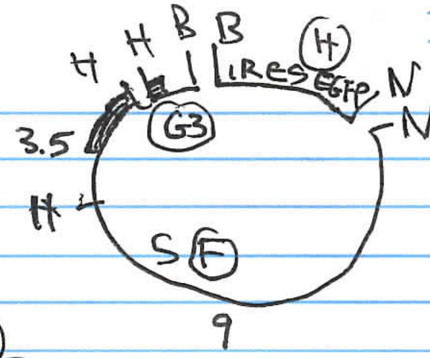
37° 1.05 pm

Take 10 µl 10X R1 salts +36 µl ladder + 1 µl HindIII (0.5) + 2 µl NotI (5) Lot57 Lot48
→ 15µl + +5µl H'143 mini ⑤ + 10µl 0.001M EDTA
② 15µl + 15µl H'143 mini ⑧ ⑮ 37° 5:25pm – 7:30pm → Cold

Conclusion #⑧ is correct. ✓✓✓✓ streaked out 11am. Tues 1x Amp NZY.

(1) On 1:28pm Off 1:58pm 1.5% H X 1 RNAse 10V/cm

Sat. Oct. 17th



Third assembly

Dye / AgMM / H'113 N H / 1 / 2 / 1 / 1 / 23 / 24 / Acr MM / W / Dye

H'143 mins H+N (1–23)

Take ✓ 7.5 μl H'125 (H)
✓ 7.5 μl H'133 (G3)
✓ 10 μl H'141 S(F)
✓ + 2.5 μl 8M NH_4Ac
✓ + 56 μl EtOH

Freeze. Cent. Dry. Back into 15 μl 1X p99 ✓ ✓

as H'143 LIGN

3+12 ✓ ✓ | + 1.5 μl T4 ligase dilute Lot 26

PRE H'143

to 15° 2:23pm to 4:40pm
RT 4:40pm to 5:30pm
37° 5:30pm to 6:10pm → to cold ✓

3+12 POST H'143 ✓ ✓

#8 streaked out 11am Tues.

On 9:59am Off 10:20am 1.5% H X 1 RNAse 10V/cm

Dye / W / AGMM / W / (5) (8) (15) H'143 mins H+N / W / ACR / W / Dye

Transformation

H'35 DH5α	80 μl ✓
H'143 ligⁿ	3 μl ✓
0° 6:13pm	to 6:48pm
✓ 90 sec	42° (2/4) ← larger (1)
Plated Rept. 20, 5	20/16, 5/16
to 1X Amp NZY	↖ (11/20)

37° 7pm to 1:20pm

Note linearization with Kpn I will not work, since there is a site in (H). Pvu I could be used to replace the linearization Kpn I – it does not cut in Hp plug, according to Randy. Check on final colonies.

143 Mins (1) – (24) to 37° at 6:35pm 1X Amp NZY from (5/16) ~~20/16~~ #(5)
Digested H+N 3 μl + 40 μl & ran gel (1) ~ 9am ∴ up to 30 μl final.

1) Excise heart & dissect out coronaries with the Gomez HCl method. Stain for lipid or assay some transcription factor.

---

2) Consider cell type to be determined by switching on/off states of large # of independent explanations. Find a set of cells that can interconvert by receptor/ligand states (or).

~~5) If pnNI works this problem; is frequency not promoter.~~

Sat. Oct. 7th

On cell metaplasia / metamorphosis

Our renin cell # work emphasizes the persistence or recruitment of renin-producing cells in the afferent glomerular arterioles. Metaplasia has been previously described in many systems. One that seems very relevant to our work is the metaplastic recruitment of virtually all cardiac myocytes to the production of ANF a variety of procedures (Michel et al., including Pierre Corvol. Am. J. Physiol. 251 #890-896, 1986). Possibly I can test the existence of two interconvertible steady states by inducing ANF and using whole ventricles & watching it reversion :-

Normal → Gneral → Normal
↑ Metaplasia ↑
Aorto-caval fistula (2 wks) or deoxycorticosone acetate-salt (3 wks) — Ligation of fistula & removal of DOCA.

The steady state levels of suitable transcription factors could be assayed.

Testing the same procedure in NPRA +/- and -/- mice would be informative & maybe more extreme.

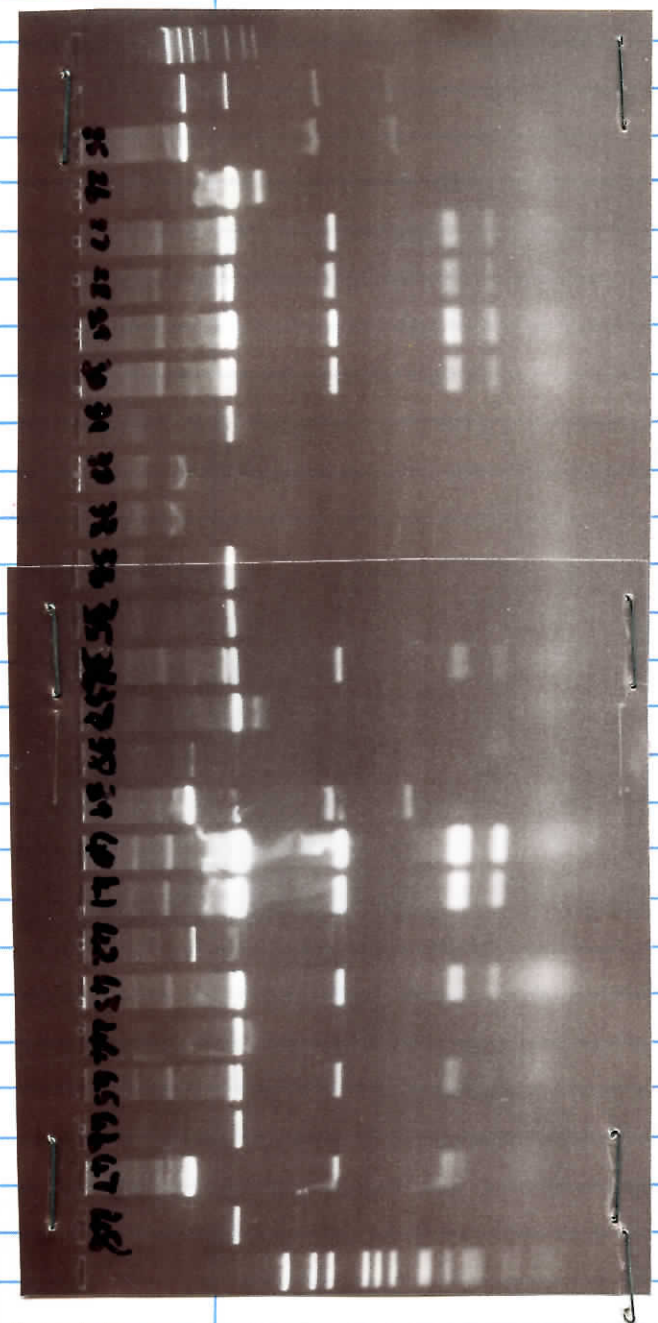
Nov. 12th

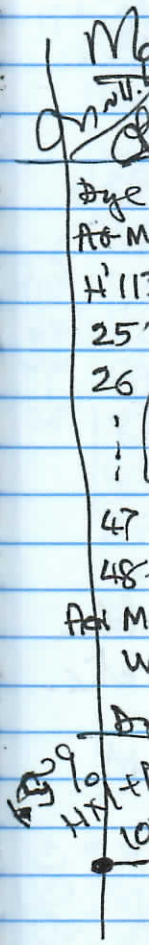
Additia

Since templates are only single molecules, may be able to count molecules / cell rather than molarities. Also a tendency in the literature is to assume flip-flop inducing ligand conc. varies between cells. May be packs to work on Poisson probabilities.

Oligo Name: H'146 KpnI-PvuI

lc:	G A C	G A T	C G T	C G T	A C
l: 14					
57%					





Tue. Oct 20th

H'143 minis → #8 (Kpn) #2 → (Kpn) + Pvu I

Ex Kpn I

GGTAC X GACGATCGTCGTAC C

GACGATCG Pvu I

Single Kpn cut & Pvu I test.

Take 10 μl 10x R1 buffer
82 μl ddw
~8 μl (whole) of H'143 mini #8

15 + 1 μl (5u) Pvu I Lot 25

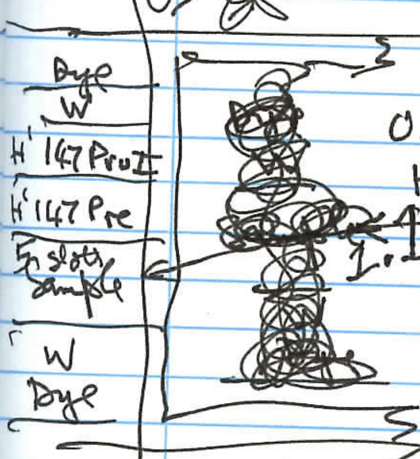
+ 1 μl of Kpn I /10 Lot (1u/μl)
1 μl 10x R1
1 μl Kpn I Lot 48 10u/μl
8 μl ddw

H'147 PRE | H'147 Pvu I | H'147 Kpn I (Prewarmed)

37° 2pm → 6:20pm, 32 min 36°
80°C for 10 min → to cold

ON 7:20pm 9:0am

Margin: Dye, Ad MM, H'113 NH, 25–48 H'143 N+H 2 μl, Ad MM, W, Dye, 1% + RNAse 10V/cm

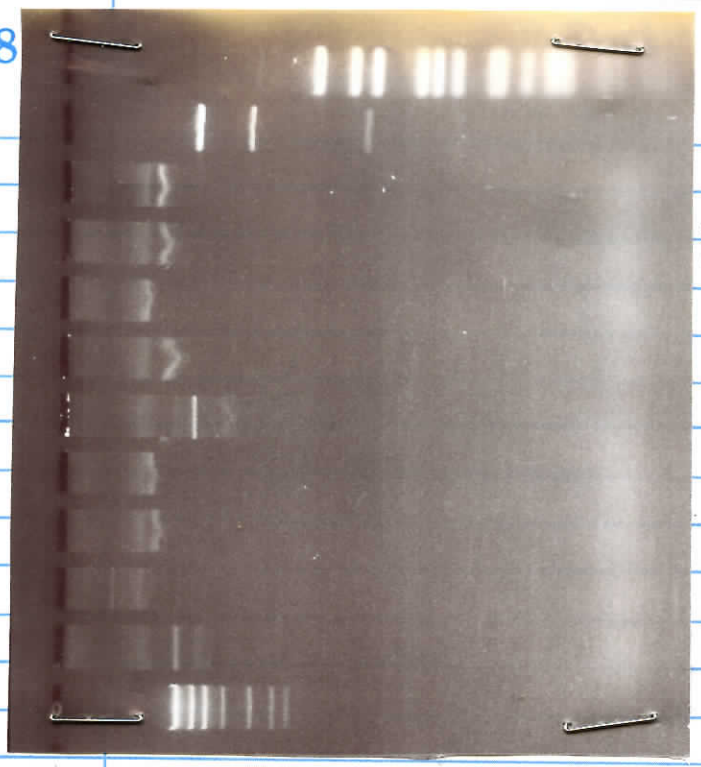


0.8%
14x4
1.1V/cm

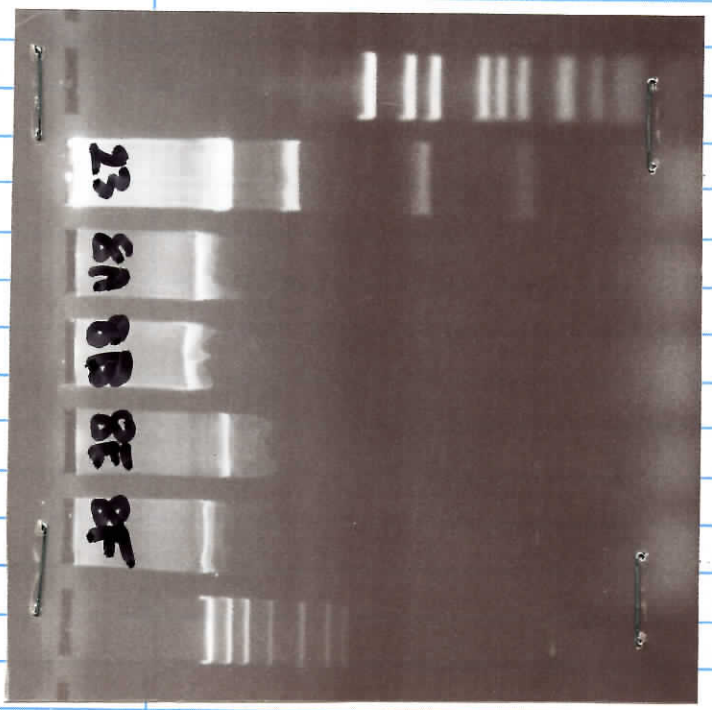
Rough expt.

H'145 minis 25–48
37 9:38pm – 1:58pm
52 μl 10x R1
450 μl ddw
6.5 μl Hind III lot 57
13 μl Not I lot 45
20 μl + 2 μl each mini

Unfortunately — a dried up area in important part of gel!
Results are uninterpretable — Regrow & repeat
→ Only (38) looked possible &, as usual, it is the worst grower.



① Conclusion Disappointing — only (E) looks anything like correct, but note that (E) digests with KpnI (& probably PvuI also). Turned gross ext over to Danby & repeated H+N on several at 3.5 × amount.



② Conclusion This problem is partly electrophoretic. Run in 1.5% H×4 at lower V/cm — or N.M. do φoH/HOQ

H113 NH low

11K43

On 12:50pm
Off 1:20pm
1.5% H×1+RNAse
10v/cm

Wed. Oct. 21st

#8 (cont'd)

Dye (1)
W
AGMM
8A
8B
8C
8D
8E
8F
8F PvuI
8F PstI
8F KpnI
AGMM
W
Dye

8A ... 8F picked from streak of #8 H'143 Minis at 12:30pm ⇒ 1×Amp N24 Various ages. This will check stability etc.

H'149 H+N

gave me enough to go on with for Kpn → Pvu but from a good sized colony

9a.15am Replica of (E) is very poor. (B) & (F) are the best, but relative growths in liquid cultures are all v same.

Take 20µl 10× R1 buffer
+ 180µl ddw
60µl + 6µl H'149 (8A)

+ 1.5µl HindIII Lot 51 (30u)
+ 3µl NotI Lot 48 (30u)

20 H'149 PRE (F) + 1µl (5u) PvuI Lot 25
20 H'149 PvuI + 1µl (5u) PvuI Lot 25
20 H'149 KpnI + 1µl (5u) KpnI Lot 48

A ..... F + 2µl

37° 10:52am → 12:30pm

Used (F) for these

Dye
AGMM
23
8A 50/50
8B
8E
8F
AGMM
W
Dye

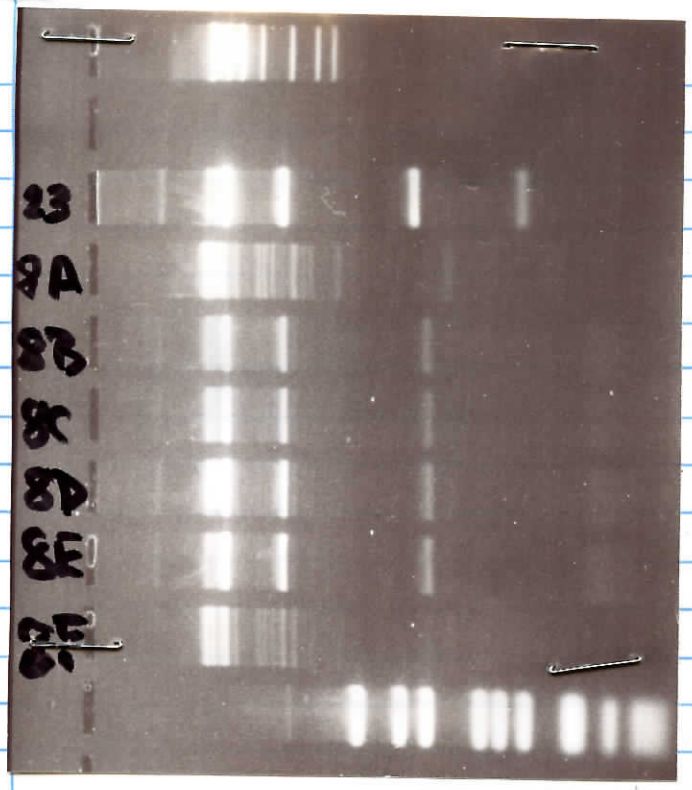
H'143
H'149 H+N

Turned over growth to Dusty – but one more left on 8E & F plus whatever.

Take 10µl 10× R1
+ 36µl ddw
+ 1.25µl HindIII Lot 51
+ 2.5µl NotI Lot 48

9	9	9	9	9
+ 9µl H'143 #23	+9µl H'149 8E	+9µl H'149 8F	+9 H'149 8B	+9µl H'149 8A

37° 3pm to 7pm – to cold.



D. Carchesia: All are ok probably but (A) & (F) are partial. Certainly (B) - (E) are good.

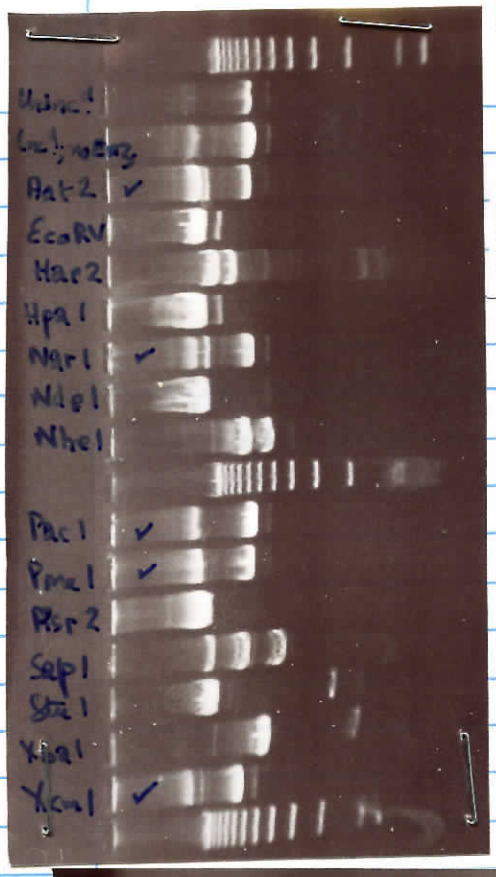
On 1:43 pm
Off 2:13 pm
1.5% 9 H+1 RNAse
10V/cm

Fri. Oct. 23rd

More 8A - - - F + 23 6809

Dye
W — H'143 #23 + H'149 8A-F 1x PhOH/HOQ 2xCIAA
AGMM after addition of 400μl 8/10 M NH_4Ac. EtOH pptd. Freeze
W 23 Cent. + back into 30μl 0.001M EDTA (with black tops).
8A
8B Take ✓ 10μl 10x R1 salts
8C ✓ 58 μl ddw
8D ✓ 2 μl HindIII Lot 51 (5u/sample)
8E ✓ 4 μl NotI Lot 48 (5u/sample)
8F ✓ 9μl to each plus 9μl of H'143 #23 + H'149 8A-F
AGR MM 37° 11:25am — 1.30pm
W
Dye

GOOD NEWS! Perfectly stable — just a very poor grower.
Can do some sequence.



(1) Randy's gel (#66)

Ex KpnI PacI

GGTACCGGTTAATTAACCGTACC

CCATGGCCAATTAATTGGCATGG

20457

Oligo #:

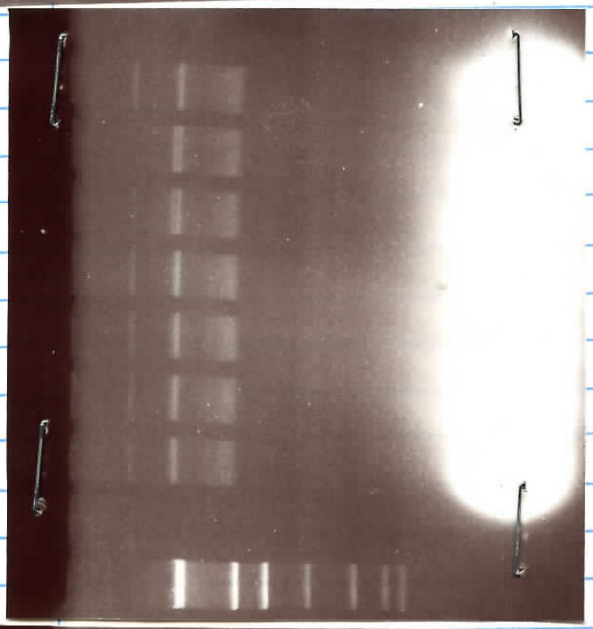
Date made:

LENGTH: 16

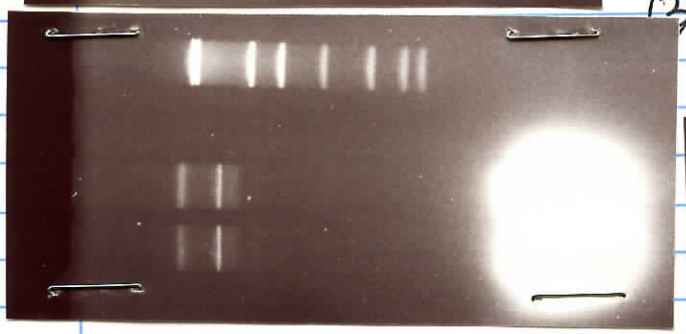
% G+C: 38%

5' Oligo Name: H'152 ~~KpnI~~ to PacI 3'

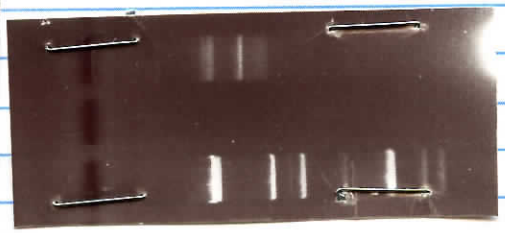
G	G	T	T	A	A	T	T	A	A	C	C	G	T	A
C														



(2) Conclusion PacI & PmeI conditions probably nick the DNA. Kpn no sign of action. Recover & try more Kpn. Make a ~~Kpn~~ → PacI oligo.



(3) Looking better, could try this & risk 2X on cac?



(4)

On 10:07pm
Off 2:?4 HX4
0.8% max

Dye
W
Pre
PacI
PmeI
1
2
4
8
W
AgMM
W
Dye

(2)

Sat - Oct 24th

Single cut KpnI on #8

(1) Randy has tested a variety of enzymes that will not cut Bst X1 IRES EGFP XhoI Not for ability to cut VIII.29 (see left). Let's try PacI & PmeI (both 8 base cutters) & work on single KpnI site.
Pool H'149 B through E as [H'153 8BE] ~100 µl available.

Take ✓ 21 µl 10×R1
✓ 159 µl ddw
✓ 30 µl H'153 8BE ≅ 3 µl / usual rx

Prewarmed
+ ½ µl (5u) KpnI Lot 48 10 u/µl

✓ 15 µl + 1 µl (4u) → [PacI] Lot 32 4 u/µl
✓ 15 µl + 1 µl (4u) → [PmeI] Lot 13A 4 u/µl
✓ 15 µl → [Pre]

40 µl [1 min] | 40 µl [2 min] | 40 µl [4 min] | [8 min]
Rest to 80-85°C ≥ 5 min

32° 11:43a — Not a good test – sh'd use K+

On 3:5?pm
Off 4:56pm HX4
0.8% max

(3)
Dye
W
AgMM
W
2²
8² } H'153 KpnI
W
Dye

[Pool as appropriate; ligate chosen linker; gel purify; transform!]

No KpnI cutting apparent. Recovered 80 µl from the 1, 2, 4 & 8 min digests. Tried 1/2 µl KpnI Lot 48 (10 u/µl) for 2 and 8 mins. ✓ ✓

[H'153 2²]
[H'153 8²]

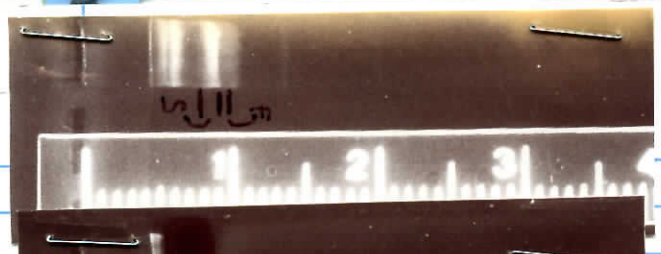
On 10:50am
Off 11:?0 HX4
0.8% max

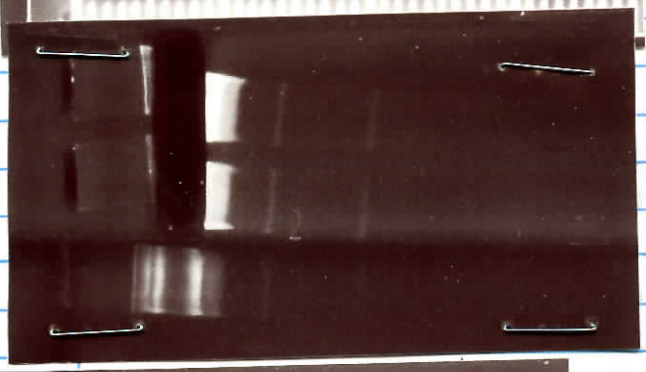
(4)
Dye
W
H'153 #8K
W
AGMM
W
Dye

Take 20 µl 10×R1
110 µl ddw
Whole (~70 µl) H'153 8BE [#8 Pool] ~190 µl
Prewarm 37°
+ 3 µl KpnI Lot 48 (10 u/µl)
37° 8 mins. ≥ 5 min 80-85°

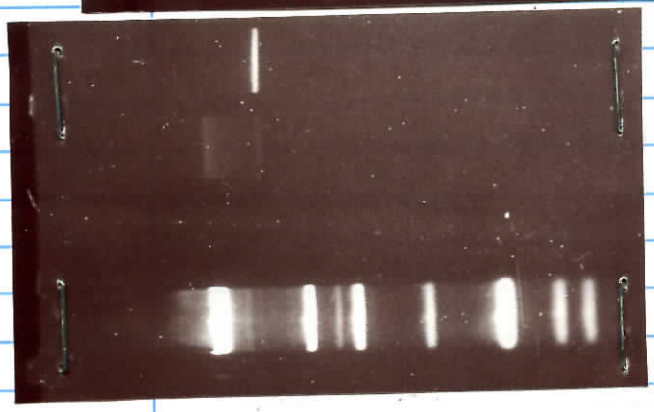
→ [H'153 #8 K]

Conclusion: Just about usable with total pool.





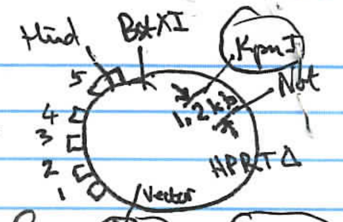
① Not a good cut – gel was cracked



② Not much, but v. nice.

Mon. Oct. 26th

#8 ~~Kpn~~ → Pac I



Take whole ~200μl H'153' #Pool
+ 1μl (10μg) lab stock RNAse

55° 8.44 am to ~9:25 am. 1X ΦOH/HOQ. 2xCIAA

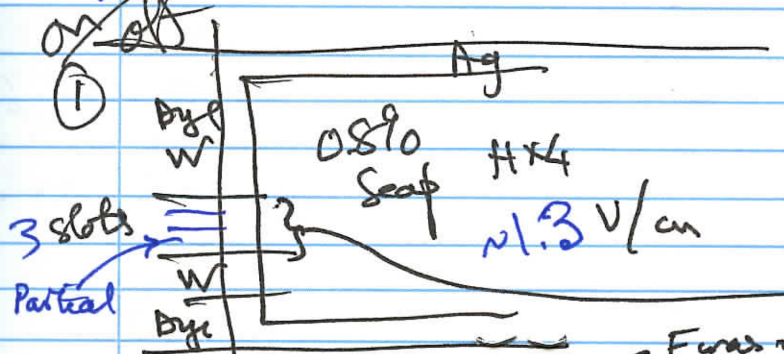
210 + 21 [illegible] μl 8M NH_4Ac + 500 μl EtOH

Freeze. Cent. Dry. Back into 30 μl 10x p95 buffer
+23μl ddw + 1 μl H'152 ~~KpnI~~ to Pac I oligomer
+ 3 μl T4 ligase dilute hot 67

H'155 #8

15° 4pm to 4:45pm, R.T. [illegible] to 5:42, 37° to 6:15pm

6:30pm on
off



F was ~50 mg of gel
S was ~100 mg of gel

On 11.45am
Off 1:12pm

Did best cuts I could – not good – but F may be workable.

Recovered into F ← S ← strange! ppt: looked like mini preps!!
30μl 50μl 0.001M EDTA

Dye
W
H'155 F
H'155 S
W
AGMN
W
Dye

0.8% H x4
Seaplaque
at 12:50 to max

H'155 F | H'155 S

H'35 DH5α	80 μl
H'155 F	15 μl + 1.5 μl 10x PBS
3.07 [illegible]	4:55
90 sec	42°

Plated 1x Amp NZY Rest 20, 5, 20/16, 5/16
37° ~5pm

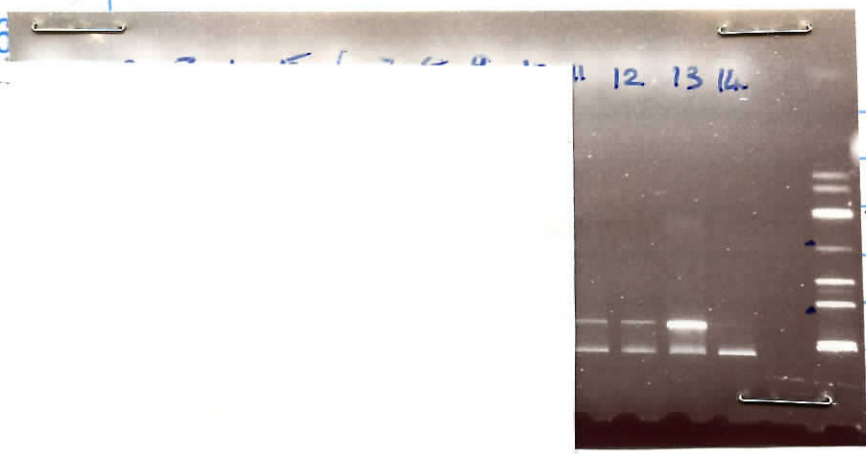
Wed 12pm

9.45am Wed. Repeated with rest of F 280μl – but no PBS
7 x 10μl plates

10:35am 37° at 10.45am → 10:30am ~ 16 1 or 2 large

Nocolonies

156



(1) Conclusion: No sign that PacI cuts, altho Not (presumably) cuts many, including #23. #13 is a good grower. Repeat with better buffer for PacI & longer times.

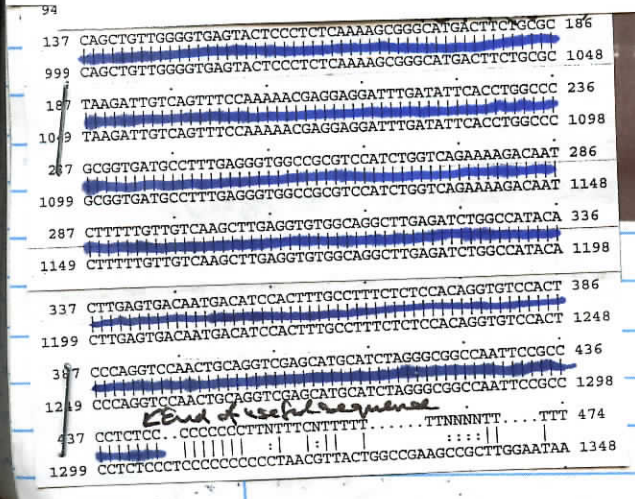
Streaked out H155 #13 To Randy Mon. 10:30 am & at 6:25 pm H155 #13 Too.

2B Conclusion - all 9 are identical with 2 sites minimum. Measured sizes are 8.6kb & 6.1kb

Some question if Xho cuts at all visibly since only 1X cut in 23 with X+P

2A

Test Xho I on #13 & on #23.

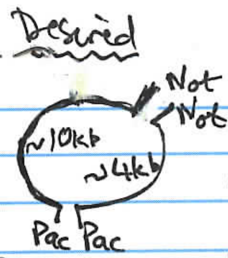


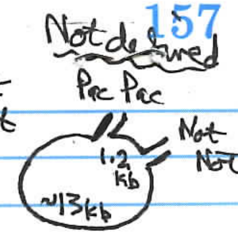
End of useful sequence

2:22pm
Off 3:32pm 0.8% Hx4 + RNAse 2µl/10ml
0.8% Max

Thurs. Oct 29th

H'155 minis





Dye — W
H'143 #23
1
2

H'155 minis (1) — (14) 1x AmpNZY to 37° 11:15am
8:50am

Processed to 50µl. H2O/Q/CIAA

Take ✓ 30µl 10x EcoRI buffer
✓ 243µl ddw
✓ 20µl PacI lot 32 (4u/µl) 5u/sample
✓ 7.5µl NotI lot 48 (10u/µl) 5u/sample

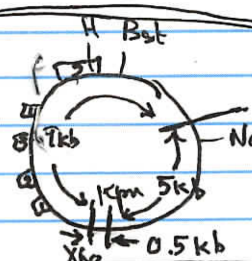
13, 14, W, AGMM, W, Dye

20µl aliquots + 2µl of minis. + 2µl 23 H'143
Time was not recorded ~ estimate 37° at 12noon 1 1/2 hr. 1:02pm
Back to 37° 2:22

4:45am 0.5% Hx4 R
Off 0.8% Max

Sun. Nov. 1st

Repeat digests



Desired K+X (single)
5.5 + 9
Undesired K+X (single)
0.5+14
Two Kpns (no change)
0.5 + 5 + 9

Dye, W, 2A, Pac

Could use XhoI to linearize (see H'10) since it does not cut in fragment(s) (G) + (H) & takes out only 500bp of 5' homology.

Pac sh'd cut singly

Meanwhile for checking current minis, repeat PacI with new enzyme, fresh buffer & overnite, also check with KpnI

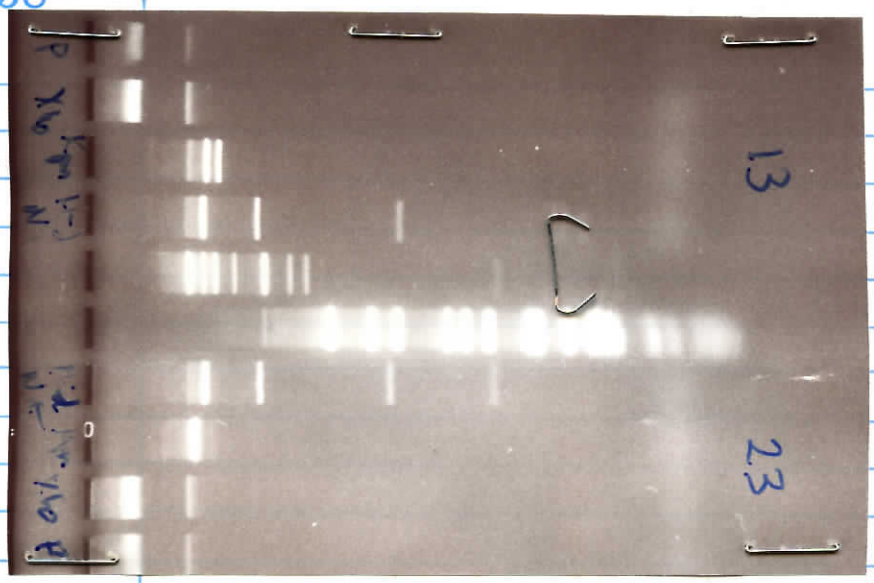
AGMM W, X+K, 2A, X+K, W, RMN, Dye, Pac

Take ✓ 22µl NE buffer 1
✓ 22µl 10x BSA
✓ 154 µl ddw + 11µl XhoI (110u) lot 35 (10u/µl)
✓ 11µl KpnI (110u) lot 48 (10u/µl) 5u/sample

20µl + 2µl each H'143 #23 & H'155 (2)(3)(5)(8)(9)(10)(11)(12)(13)
4 4 4 4 4 4

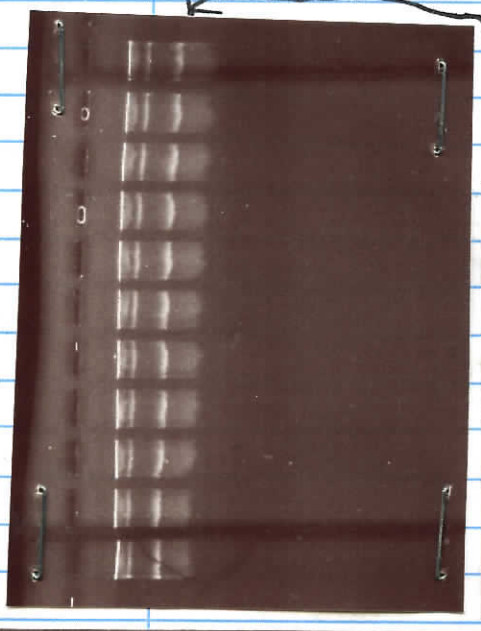
Take 22µl NE buffer 1 except
✓ 22µl 10x BSA
✓ 14µl ddw
✓ 28µl PacI (110u) lot 47 (4u/µl) 5u/µl — Fresh lot

37° 5:50pm

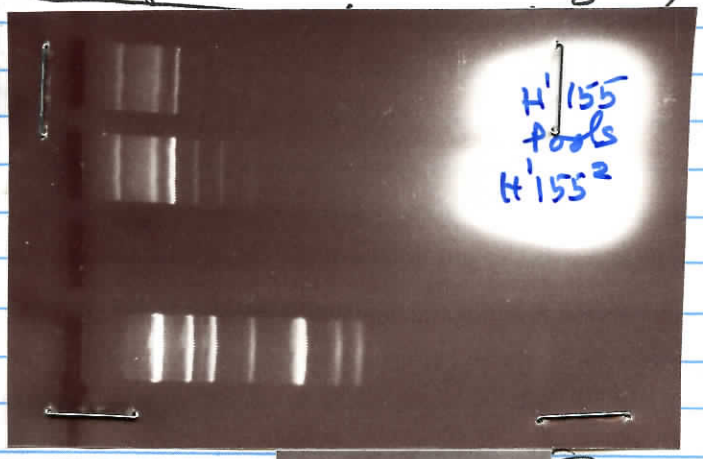


① Conclusion:
#13 is fine! ~~that~~ 2 Kpn cuts & no XhoI
- so ate Xho for site change, not PacI.

#23 has no XhoI, 1 KpnI & usual next.

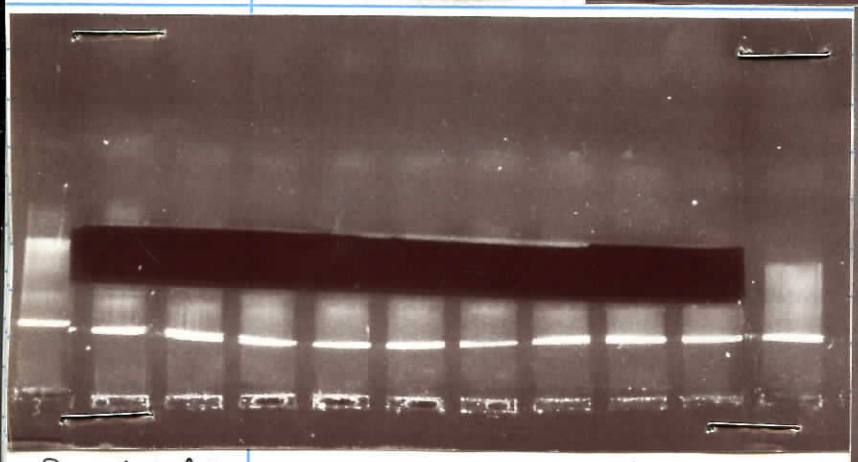


② Not enough cutting. Wait for better DNA from Pappy
May have been some single.)



③ Much better!

③ 2:45 pm
ON
Dye
W
H'159 Pool
H'159² Pool
W
PEG MM
W
Dye



⑤ single
Dye
W
H'159 Kpn SC
W
MM
W
Dye
2:50 pm
ON
3:35 pm

④ Terrible s.p. - scrap this seaplaque. Try best I could.

⑤ Usable product anyway!

Mon. Nov. 2nd

Checks on #13 (& H'143 #23)

(1) On 3pm / Off 3:35pm / 1.5% H×1 R / 10V/cm

Dye, W, Pre, Xho, Kpn, H+N, Ag. MM / Act. MM, H+N, Kpn, Xho, Pre, W, Dye

#13 Take 14 µl 10×RI salts 7×7
100 µl ddw 50×50

57 µl — 57 µl

6 µl H'155 #13 — 6 µl H'143 #23

20 20 20 — 20 20 20

1/2 µl XhoI (10U) Lot	1/2 µl KpnI (10 U) Lot 48	1/2 µl HindIII 10 U Lot 51; 1 µl NotI 10 U Lot 48	1/2 µl XhoI	1 µl KpnI	1/2 µl HindIII 51; 1 µl NotI Lot 48

37° 1:35 pm — 2:35 pm

H'159 KpnI to XhoI

Oligo # 20548 5' Oligo Name: H'159 KpnI → XhoI 3'
Date made: 11-2 GAC TCG AGT CGT AC
LENGTH: 14
% G+C: 57% 5' Oligo Name:
dC CPG

(2) On 6:15pm / Off 9:30am / 0.8% H×4 / 0.8 V/cm

G GTAC | GACTCGAGTC | GTAC | C
C | CATG | CTGAGCTCAG | CATG G

Dye, W, slots, W, Dye

Take 20 µl 10×RI salts
164 µl ddw
16 µl H'155 #13 mini
Prewarm
2 µl — 1 µl KpnI Lot 48 (10U/µl) (10 U)

40 µl	40 µl	40 µl	Rest
1 min	2 min	4 min	8 min

→ Pooled

H'159[2] Pool

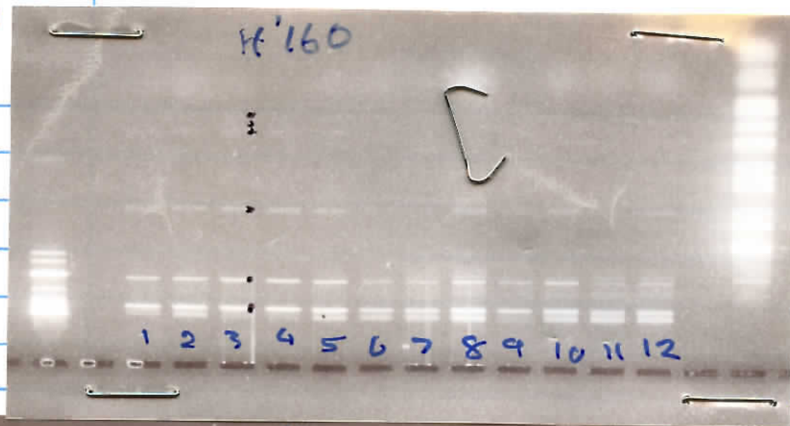
H'159 Pool

80°–85° ≥ 5 min.

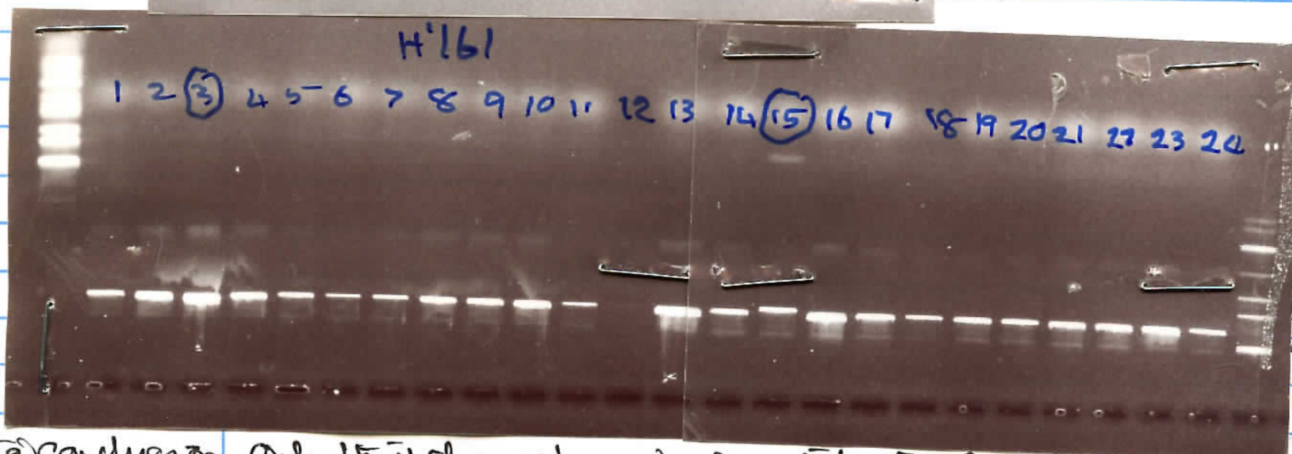
Post-heating vol. 25 µl.

(3) repeated On 4:40pm / Off 9:00am / 1.3 V/cm

Recovered into 50 µl of H'159 (Kpn Single)(cut) — but wasted some.



① Conclusion
All about equal & okay catalbery growth. Pooled as H'160
#13 pool



② Conclusion Only 15 is changed — & wrong site. Pool rest & continue.

← Randy Thrasher α83 gel. PvuI looks good for #13

H'161 Pool

① 1:40
2:05
1.5%
Dye
W
AG
2 H'160
11 min
12
ACR
W
Dye
1:2
250
Dye
W
ACR
1
2
H'161
23
24
AG
W
Dye
0.8%
HxLR
Max

Wed. Nov. 4th

More with H'155 #13

Picked 12 nice sized single colonies from H'155 #13 streak as minis 1–12 H'160. Randy finds 2 enzymes that are suitable for linearization of current construct. So concentrate on growing the least. ? on about 12 noon. Off 9.0 am — poor growths in general

Kpn I to Xho I on #13

Take 10 µl (~45%) of H'159 KpnSC
+ 1 µl H'159 Kpn I → Xho I oligo
+ 1.2 µl 10 × T4 ligase buffer
+ 1.2 µl T4 ligase dilute lot 67
to cold room 4:45 pm to 1 pm

H'161 4.16 N

→ Checked temp. ≥ 38.5°! Growth on replicas is uniformly good. Processed to 50 µl. HOQ/CIAA

Take 26 µl 10×R1 2.25 µl d d w 3.25 µl Hind III 6.5 µl Not I
20 µl aliquots 2 µl of minis H'160 37° 11:20 am to Past 4.25 pm

Lot 51 Lot 48

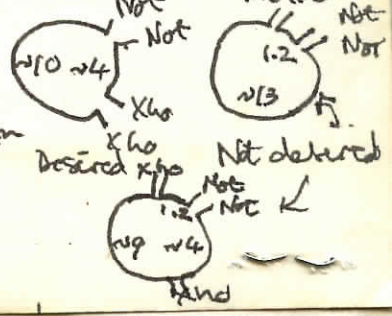
Transformation		DH5α	80 µl
H'161	11/4	H'161 4G N pm	5 µl (no heat, no dilution)
		0°C 1:05 to 1:45 pm	N20 6
		90 sec 42°	Rest 20, 5, 20/16, 5/16 1×AmpNZY 37° 2 pm 12 noon
		Plated	

Suspiciously high? too much single streaked.

Minis H'161 #1–24 1×NZY Amp 37° 12:25 to 8:25 am Modest growth.
Processed to 50 µl HOQ/CIAA Take 5 µl 10×R1 4.5 µl d d w 6.5 µl Xho I 13 µl Not I
3 plexed. 3rd 6:13 am – 1:30 pm 20 U/µl Lot 35 100 U/µl Lot 48

(2) Conclusion: 23/24 are unchanged – as expected – 1/24 is unclear change. ∴ screen more from H'161 ligation. H'161 #3 is a good grower. 37° 6:15 pm

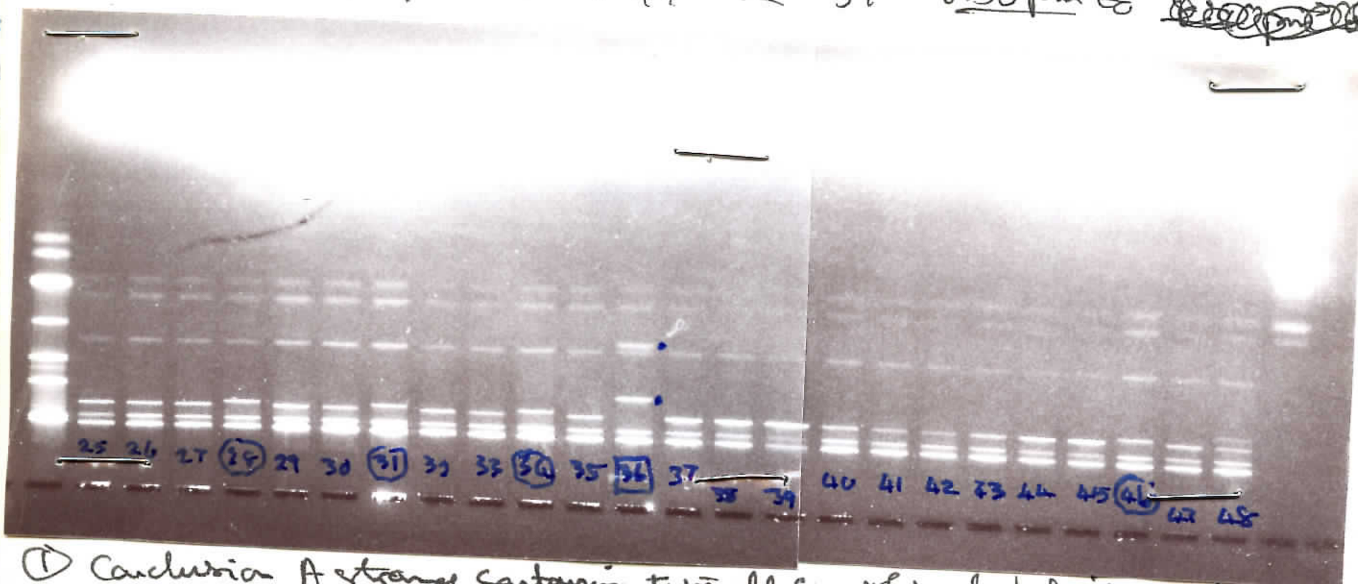
Mon. Nov. 9th picked minis H'161 #25–48, 49–72



CG2 Tues. Nov. 10th Kpn → Xho I #13

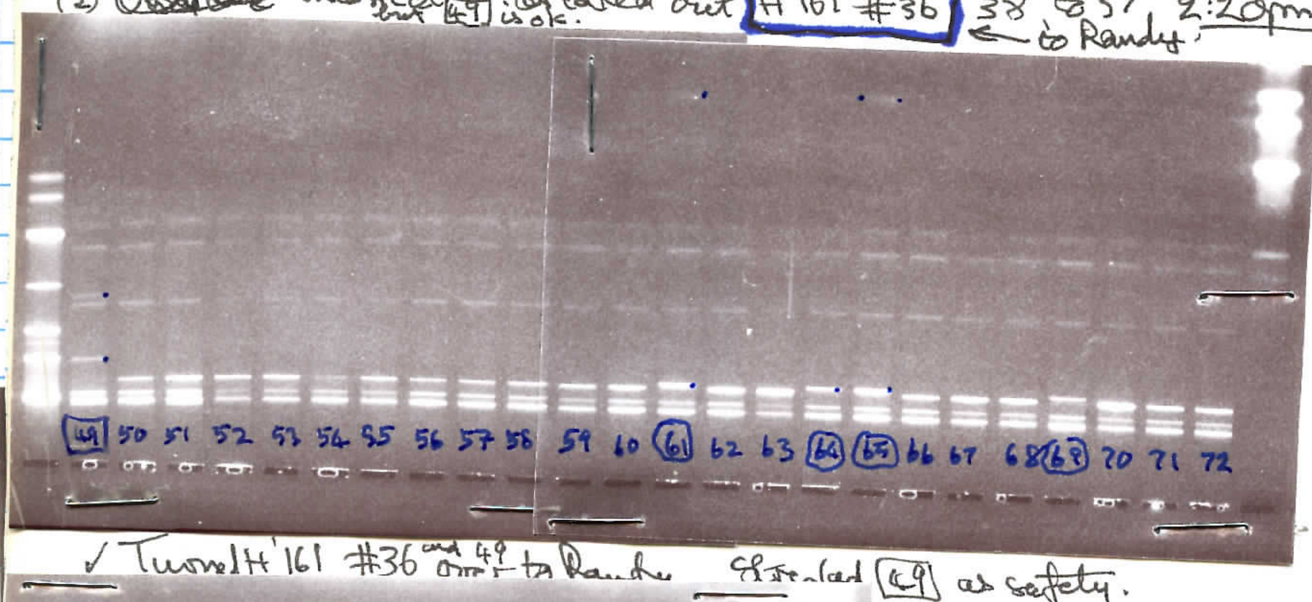
Mini 26 – 48 processed to 50 µl with HOΦ/CIAA — Same problem with excess $CHCl_3$ at last stage — may be OK

37° 4:05pm 52 µl EcoRI salts — 6.05pm Mini 49 – 72 37° 6:50pm to ~~[illegible]~~ 44.9 µl ddw 6.5 µl XhoI lot 35 13 µl NotI lot 48

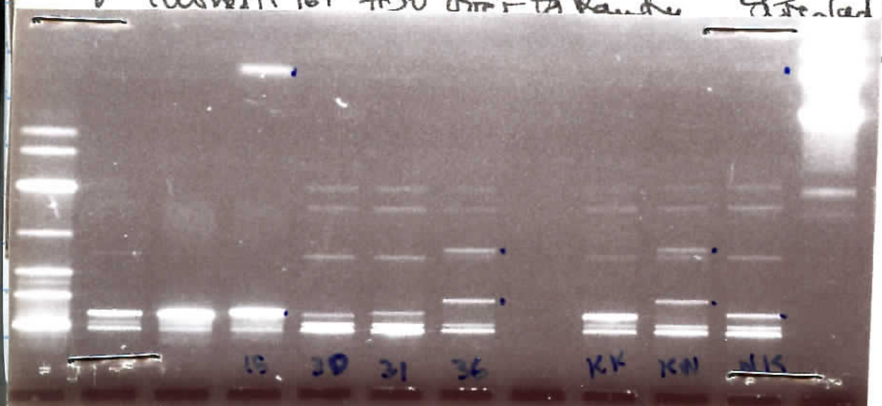


① Conclusion A strange contaminant in all samples — look for it in gel ② & scrap solution. May need to cut & retransform. (28)(31)(34) & (46) are "incorrect Kpn site", [#36] looks good. The contaminant is not from ligation since it is missing in ① – (24).

② Mainly the "incorrect" but [49] is ok. Streaked out [H'161 #36] 38 to 37° 2:20pm ← to Randy.



✓ Turned H'161 #36 and 49 over to Randy. Streaked [49] as safety.



#4.5kb
9.5

③ Conclusion No contamin^n at ligation stage.

163

Dye W AG MM 25 ⋮ 48 AC MM W Dye

(1) Vdt ↓ to 2/3 at 6:50. ↑ to max 8:05 pm off 5:25 pm

On 10:27 am off 1:20 pm ↘ 4 µl/10 ml RNAse ↙ 11:40 ↓ V to 2/3

H'161 minis Xho + Not

(2) 49 ⋮ 72 H'161 minis Xho + Not

Thurs. Nov 12th

Testing for contamination

S.H. suggests the extra bands are due to growth conditions (ssDNA or whatever) since bands are proportional to total DNA. Pooled digests (aerosite at r.t.) 38, 63 to 70 (unmodd.); 36 to 39 (correct); 28, 31, 46, 61, 64, 65, 69 (uncorrect). Using fresh Not & Xho & RL salts

Set up new digests of H'160 Pool, H'161 #15, H'161 Pool, H'161 #31, #30, #36

37° 10:52 am 20 µl → 2.5 µl each enzyme 14 µl 10×RL (fresh) 120 µl ddW (fresh), 2 µl XhoI Lot 35 (fresh) 11/12/98, 4 µl NotI (fresh) Lot 48 11/12/98

1:22 pm

Dye W AG MM

H'160 Pool
H'161 Pool
H'161 #15
H'161 #30
H'161 #31
H'161 #36
} H'163 Xho + Not

MIX ≥ 49 H'161 Unmod Pool
H'161 Correct Pool
H'161 Uncorr. Pool

AC R MM MIX H'163 Dye

Overnite at room temp from Xho Not

Boorum & Pease™

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

150 Pages	300 Pages	Ruling	Width of Desc. Space	Units Per Column
Record Ruled		H'163 Sequencing tests	H'163-1	
21-150-R	21-300-R	Record		
Columnar / Ledger / Quad Ruled		(81)		
Single Page Forms		(82)		
21-150-2	21-300-2	2 Columns	3 3/8"	10,000,000
21-150-3	21-300-3	3 Columns	3 3/8"	1,000,000
21-150-4	21-300-4	4 Columns	2 3/8"	1,000,000
21-150-5	21-300-5	5 Columns	1 5/8"	100,000
21-150-6	21-300-6	6 Columns	1 3/8"	10,000
21-150-7	21-300-7	7 Columns	1 3/8"	10,000
21-150-DEL	—	D.E.Ledger	1 3/8"	10,000,000
21-150-Q	21-300-Q	Quad-5 Sq.	–	–
Double Page Forms				
21-150-8	21-300-8	8 Columns	3 5/8"	10,000,000
21-150-10	21-300-10	10 Columns	4 1/4"	1,000,000
21-150-12	21-300-12	12 Columns	2 3/8"	1,000,000

ESSELTE

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H'163 Sequencing tests H'163-1

#110 1/100 ddW Hp 485/7 Blueprint 5 µl

Columnar / Ledger / Quad Ruled (81)

H'161 mini 36 ditto but 1.5 µl Randy's DNA H'155 #13 from d80 H'163-2 12 µl 3 µl

Single Page Forms (82)

Later thought

The faint Maxam may be F' from DH5d. The gel ③ looks as if this is correct. No contamin. effect detected at digestion stage

Conclusion 81 failed. 82 confirms expectation perfectly

— see H'156 for data.