Fri. Dec. 17th

Repeat of n 93 expt with new DNAs

Aim: To test new Ch3A (+lac) for ligation & nicks.

~~Ch3A(+lac) 391 growth DNA from Bill W. 0 µg/µl.~~
~~- called 01 Ch3A — wrecked!~~

100ml

✓ 10.9g
✓ 2.03g

Fresh Sugden's 10X R1 (+ATP)
0.9M Tris to pH 7.8
0.1M $Mg^{++}Cl_2$
with sterile d.w.
To 10ml

10mM DTT 15.4mg
+ 50mM ATP 32.0mg

FROZE as 01 10X R1
even though it has ATP in it

∴ tried straight phage 3A (available at OD 108 in ~6M CsCl) ≡ DNA
i.e. $\frac{50}{.02 \times 1000}$ µg/µl or 2.5µg/µl

Used ½ — but really 0.8

- So can try:—
6µl (≅ 15µg)
+ 54µl sterile {0.01M Tris, 0.001M EDTA pH 8.0}

Heated phage 01 } Heat to 85°C for 5 min. ← ? Too high for lin but will allow good internal sticky ending

~~+ 2.7µl 0.04M $MgCl_2$ (sterile)~~
~~+ 12µl 01 10X Sugdens + ATP + DTT~~
~~+ 36µl d.w.~~

Miles ligaschot 10/6 2.5 µl to 12.5 Sugdens 1X

~~1µl to 99µl~~
DORI /50 ~~1µl to 99µl~~
RI/100 5µl to 5µl mI EORI buffer
RI/200 5µl 5µl
RI/400 5µl 5µl

PL 2 Hin III Undiluted

03 DNA

03 DNA

Miles liga

DG RI

PL Hin
Lot 2

To 37° at
3.35

1x

6 µl Ch3A phage (OD 108)
dialysed on boiled membrane
against unsterilized 0.01M Tris 0.001M EDTA pH8
from 2.15 pm to 2.30 pm (15 min) then taken up with 50 µl
sterile 0.01M Tris 0.001M EDTA pH8
Heated at 85°C for 5 mins.

buffer

O3 DNA

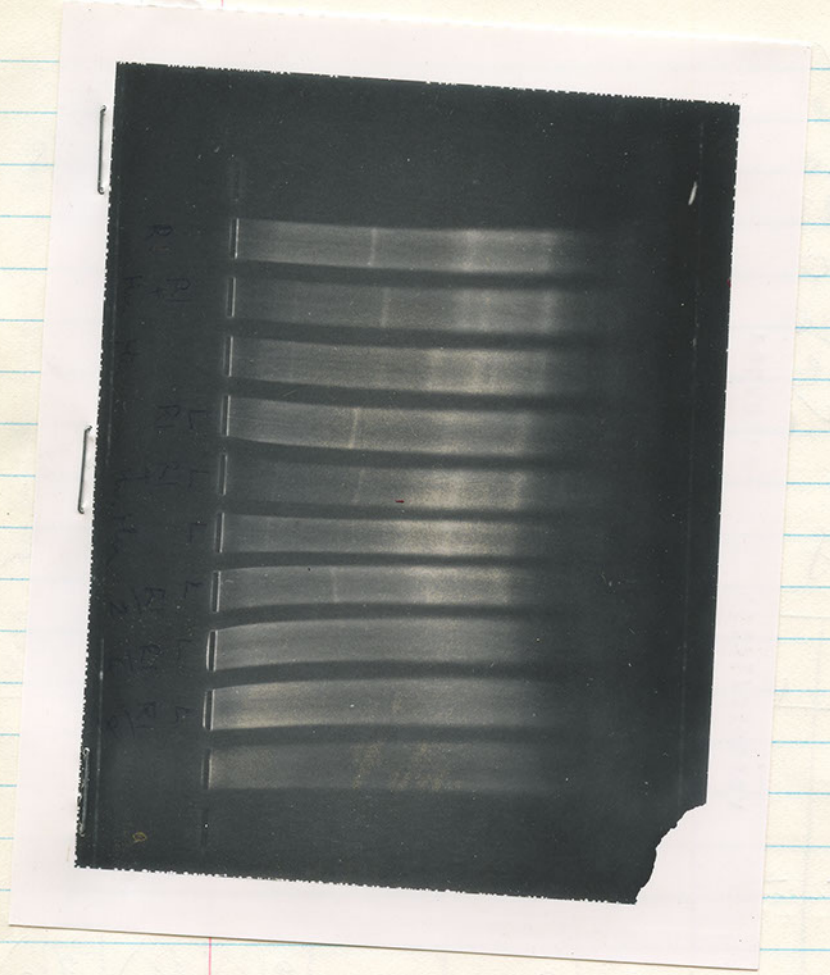
→ 60 µl to 3 µl 0.04M $MgCl_2$ sterile
12 µl 10x Sugiyama (01) + ATP + DTT
45 µl ~~sterile~~ d.w.

[Chief problem is if ligation can occur in this time]

	R1	R1 + Hin	Hin	R1 + Lig	R1 + Hin + Lig	Hin + Lig	R1/100 + Lig	R1/200 + Lig	R1/400 + Lig
O3 DNA	12	12	12	12	12	12	12	12	12
Miles Ligase 10/6	—	—	—	2	2	2	2	2	2
DG R1/50	2	2	—	2	2	—	2 (1/2)	2 (1/4)	2 (1/8)
Ph Hin I III Lot 2	—	2	2	—	2	2	—	—	—
To 37° at 3.35 pm									

Resitue as control

Stop with 3 µl { 1.3M NaOH / 0.35M EDTA
1.3 ml / 0.7 ml
2M / 1M



On 5 pm Off 10 am

0.75% Agarose
H×4
~0.75 V/cm
long gel

R1
R1 + Hin
Hin
R1 + lig
R1 + Hin + lig
Hin + lig
R1 (½) + lig
R1 (¼) + lig
R1 (⅛) + lig
Control (no enzymes)

Not bad for a first try.

Conclusions: Protein gets in the way of e.p. Control looks to be in trouble, so there may be nucleases in the $CsCl_2$ which can survive 85°C (?!). No sign of ligation. All enzymes seem borderline incomplete e.g. Hin looks incomplete ∴ R1/2 hit it work in this series.

∴ for this work, I need a micro DNA method.

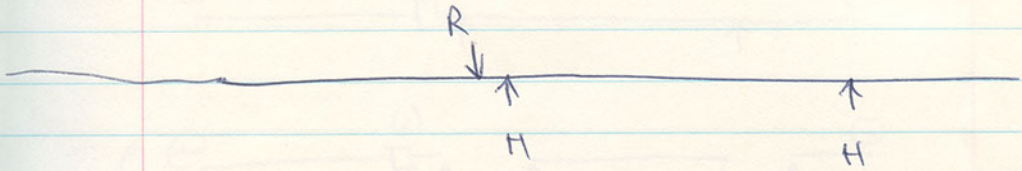
Note: Bill W. made a regular prep. of DNA with this phage prep & stated that "the phenol ate it all up" — so it has nucleases in it.

?∴ go are to a hot phenol method. Perhaps best retry this a bit more - it is promising. If ligation is needed a storage stage after heating is needed to allow sticky ends to anneal e.g. 20

Sat. Dec. 18th

Further 3A ideas

3A-lac is :—



∴ EcoR1 to completion plus any type of Hind III that gives preference of left site is a big help (e.g. 30% survival of right site with 100% loss of left would give a 30% efficient vector with no background phages).

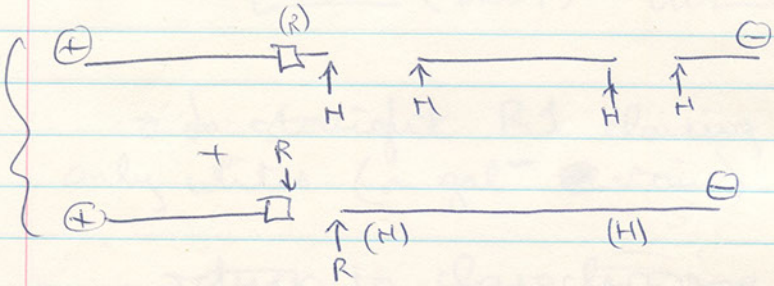
∴ try 3A-lac DNA for differential effects of salt on HindIII or of level of HindIII.

First try Sugden's buffer (+ATP & DTT) + DGR1/50 (For 30 min then) + 2fold dilutions of Hind III & ditto at usual Hind level with plus 0.1, 0.2, 0.5, 1.0 M NaCl.
(2% 5% 11% 25% vol/5 M NaCl added relative 1 vol. start)

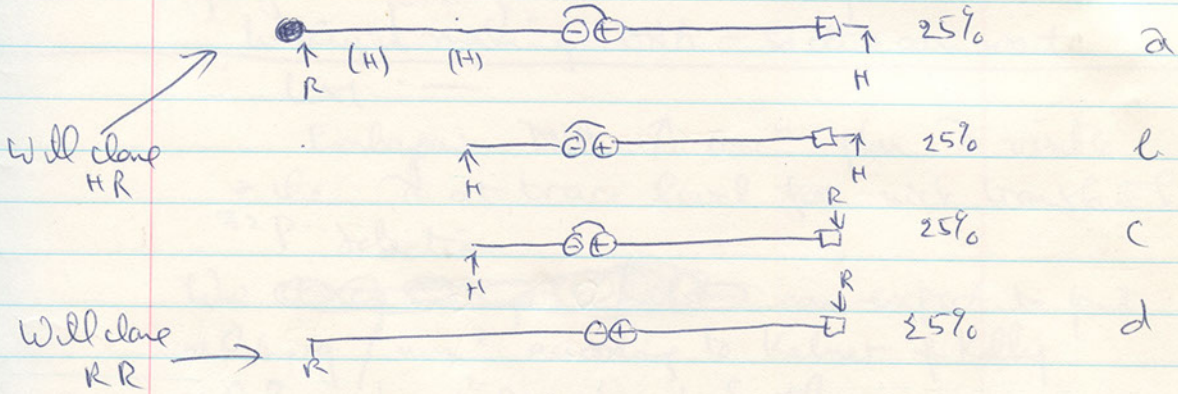
Mon. Dec 20th

Further thoughts

Ch3A - lac

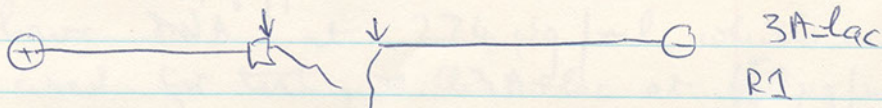


Hi temp. anneal & ligate — can use as



Trouble is background from self closure of d.

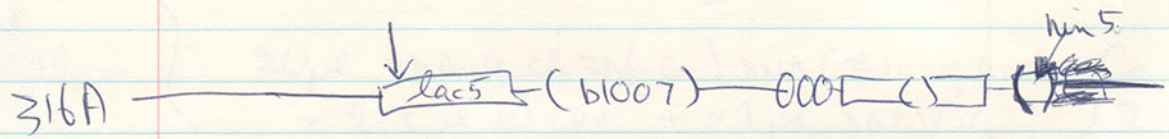
∴ better use:—



Tail with G (or C) after R1, Fill, tt in high Mg^{++} (to avoid nick) & use

this to clone PST (or any flush or 3' cell enzyme) product also tailed with C (or G) in presence of high Mg^{++}.
Result will be no self closures.

Still easiest is :—



& so straight R1 cloning & pick only whites (on gal⁻ strain)

∴ return to characterizing DNA's & improving tailing so get the method of gel hybridization working.
Wesley's making DNA — so this can wait.

∴ Next :—
Embryonic DNA ↑ conc^n plus λ visible & then λ at trace level for nick translated ^{32}P detection.
We can expect to put
~0.4 μg / mm^2 according to Kelner & Kelly
or ~0.7 μg / mm^2 according to Southern
or 5 μg / mm^2 according to our own work
i.e. up to 75 μg on our 7mm×2mm slots
or 5 μg / μl.
I have DNA at 274 μg/ml which can be used for test, & Ch3A+lac at 650 μg/ml
∴ set up up Eco R1 digest of genomic & ditto of λ & mix in appropriate proportions for test.

Mon Dec. 20th

Hybridization tests

To 37° at 11.15 a.m. ↓ 2.15 pm

200 µl (≅ 55 µg DNA) 274 µg/ml n13 DNA + 23 µl 10×R1 buffer + ~1 µl DGR1 — {o13, Gen R1}

20 µl ADA19 Ch3A(+lac) DNA (≅ 14 µg DNA) + 2.3 µl 10×R1 + ~1 µl DGR1/10 — {o13, λR1}

Stopped with 5 min at 85°C.

For test use Gen R1 diluted

1× (undiluted) → 1/10 × (with DNA buffer)

Into Control (no R1)

Add o13 λR1 at 1/1000, 1/10,000, 1/100,000 level diluted in Gen R1:

10 µl Gen R1 to 1 ml/100 DNA buffer

100 µl Gen R1/100 → 11 µl; 100 µl Gen R1/100 → 11 µl; 100 µ Gen R1/100

1 µl λR1 (λR1/10)

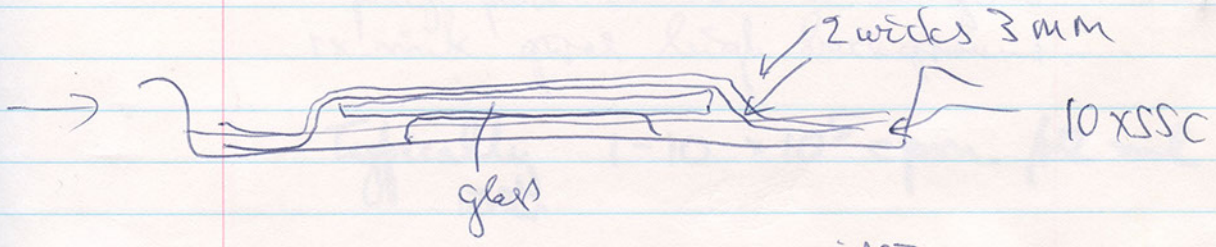
	λR1/100	λR1/1000	λR1/10,000
Gen R1	20 µl 2 µl	20 µl 2 µl	20 µl 2 µl
Gen R1/10	20 µl 2 µl	20 µl 2 µl	20 µl 2 µl

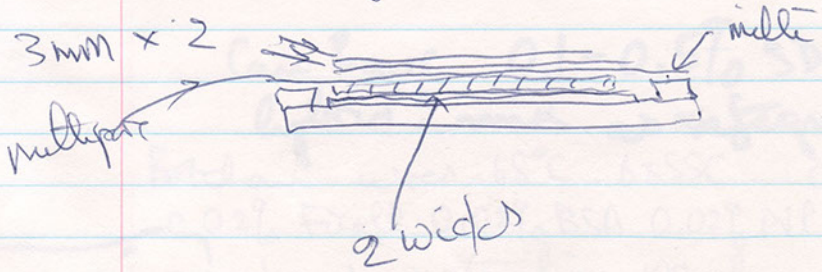
S.S. BA 85 for transfer - store dry
- works to 10^{-12-14} µg DNA in reconstruction
- ~4mm gel horizontal with agarose 65° (~0.3-0.5%) insertion with agar seal.

1.5M NaCl
0.5M NaOH } sol^n I 30-45 min with 0.7%
~60 hr 1%

3M NaCl
0.5M Tris pH7 } sol. II (critical to give large enough multipore gels v. brittle) slow shake until no visible NaOH coming out
~2 hr. 0.7%
~6 hr. 1%
overnight 2%

Neutralize. d.w. 1-2 min. then to 10xSSC just before use (a few min.)





a) Gel on 2 wicks (wet)
b) Pour 10 x SSC on top — no bubbles under gel
c) Neutralize. — no bubbles
d) 2 x top 3mm — no bubbles
e) ~2 x20 paper towels
f) Restack + ~1kg 10x10

10^6 to 2×10^5 m.w. transfer ~~time~~ 10–14 hrs.
20×10^6 18–20 hrs.
Overnight o.k. for most purposes.
~~Sometime~~ top stack replaced for 4 hr

e) Remove papers & allow (on 3MM) multipor to dry at room temp.

f) Vac. dessicate, & heat at 80°C for 2 hrs. with lid sealed to seal DNA onto filter.

Jerry uses PM mix:- 3×SSC 0.02% BSA 0.02% Ficoll 400
0.02% PVP 360

– like better than 40% formamide

– purify probe on ~~GTO~~ or on gels – crude rx. mix gives high background.

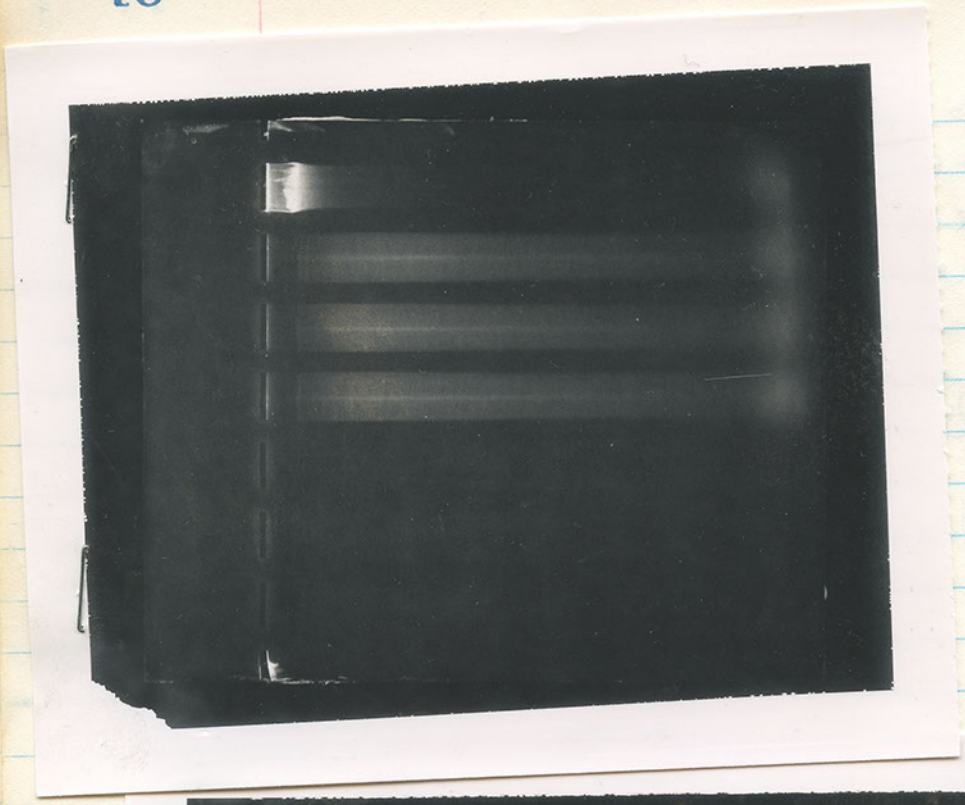
Typically 1–10 × 10^5 cpm. /1-2 ml
12 hr

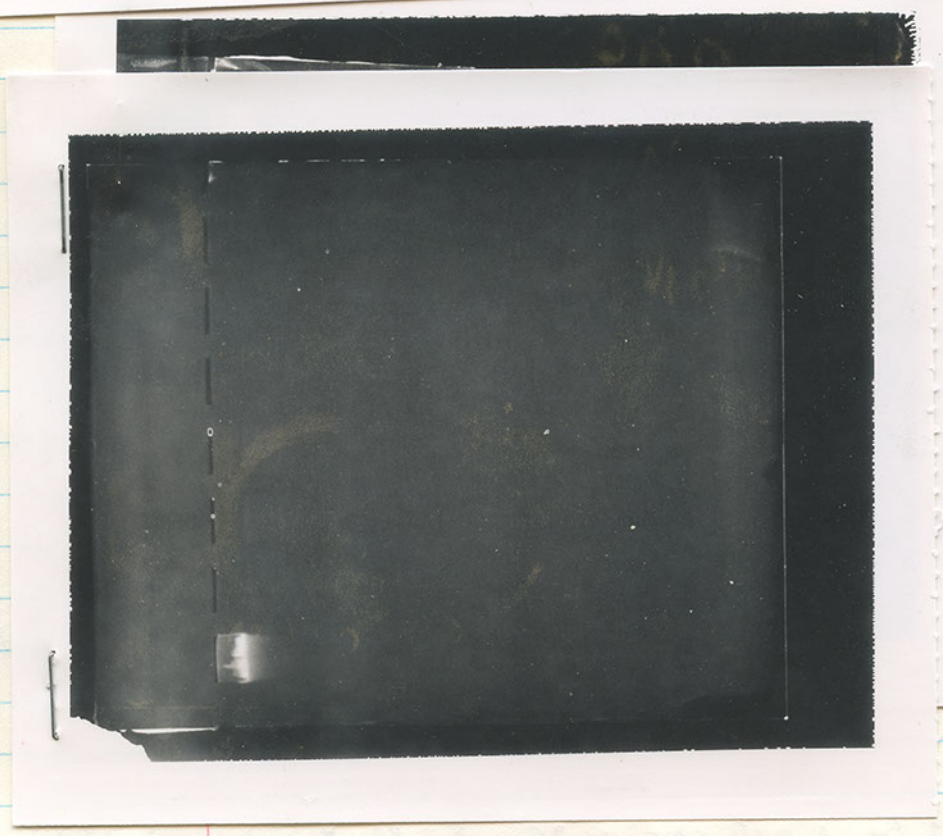
Can have 0.1–0.5% SDS in final hybrid. mix as safeguard.

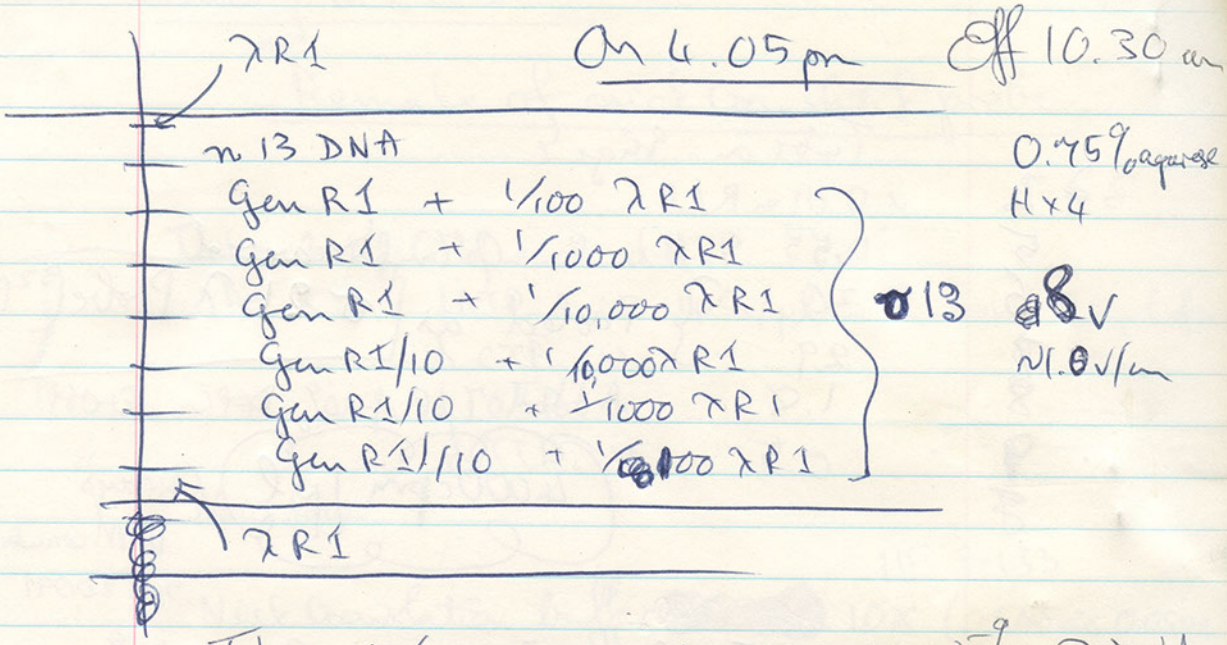
Botchan uses 65°C 6×SSC 0.5% SDS 0.001M EDTA
0.02% Ficoll 0.02% BSA 0.02% PVP
make up & sterilize 100 ml.

% hybridization sol.

60 ml 10×SSC 0.1 ml 1M EDTA
2.5 ml 20% SDS 20 mg Ficoll F4375
~37.4 ml d.w. 20 mg BSA A-4303
20 mg PVP-360
Add last
Sigma from Jerry's.







The 1/10,000 contains ~ 10^{-9} g λ DNA per slot.

Gel shows n13 DNA is good, R1 acts on it well, all is OK.

11.0am to solution I 1.5M NaCl 0.5M NaOH

11.30am to sol^n. II 3M NaCl 0.5M Tris pH7

3.0pm transferred to 10×SSC transfer system

10.0am Millipore off to 80°. Gel shows nothing until restained then high MW's are visible.

11.30am Millipore to 80°C over Drierite in vacuo

Tube on Geiger

3 ~~7~~ ~~4~~	~ 0.01 mR/hr
5	0.55
6	34
7	29
8	1.8
9	0.5

} Pooled as [~ 21 λ Probe (32P)]

0.7 ml recovered

Added 7 µl of 10% DEPC in EtOH.

4000 cpm / µl (Bray's DeMcounter) 5 µl 20,091

Total incorp. ∴ ~ 1 µG or 10%.

Cha ~ 60 µl to Jeff Ross

Tues Dec 21st

Remake of nick translation probe

Take 2.5 μCi of each dNTP

Dec. 8th dATP is 1μCi/μl ∴ took 5 μl of each
dCTP is "
dGTP is "
dTTP is "

Blow down

HF 9-133

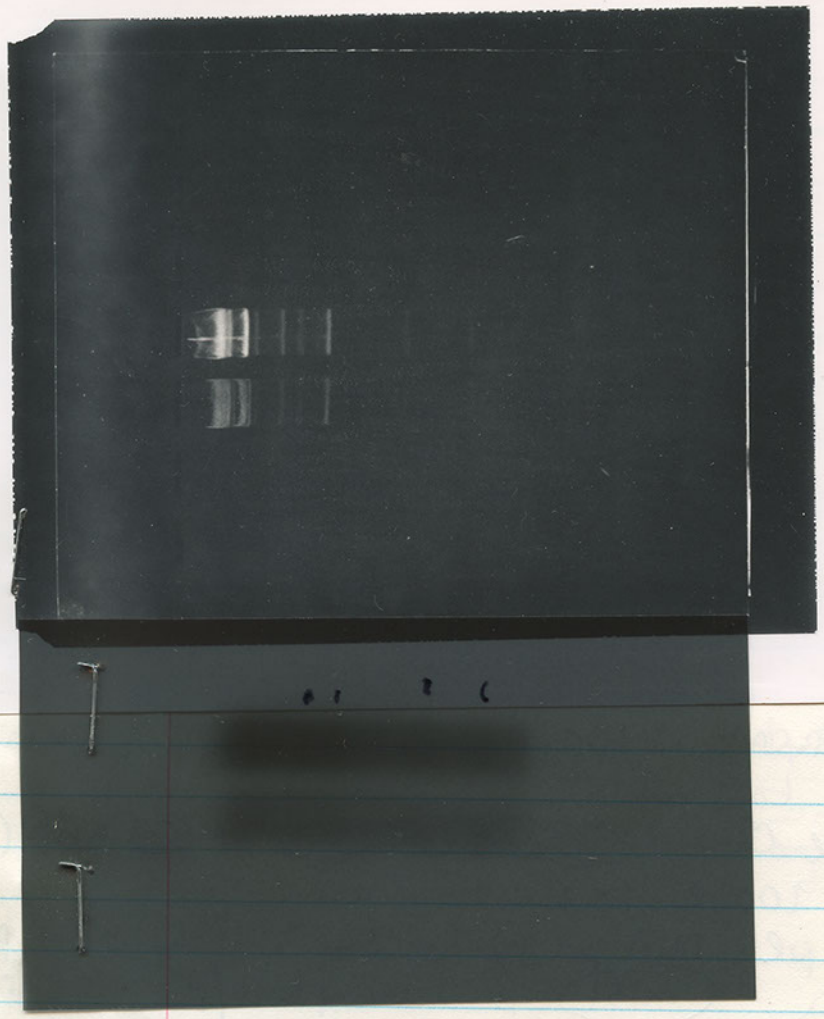
Nick translation buffer 10X (0.5M Tris 0.05M $MgCl_2$ + BSA) in 100μl portions. Add 0.7μl ME & dilute to 1 ml.

✓ 1μl Chaxan 3A DNA (0.7μg)
✓ to 100μl 1X Nick translation
✓ 2μl DNA pol I Lot 3463 P-L (DNA pol. E.coli B)
m15 } taken up to 10/μl

1pm to 37° to 1.50pm

mistake

9 100 Sephadexed
to 0.3M NaCl
0.01M Tris
0.001M EDTA
pH 7

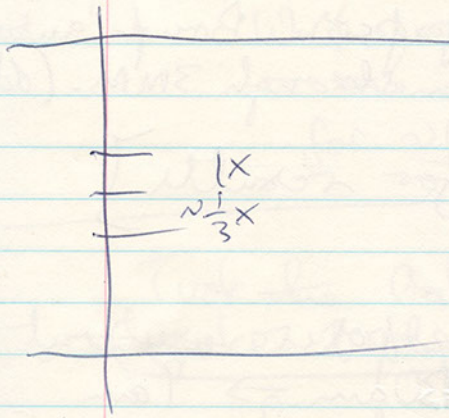


Wed. Dec. 22nd

Characterization of λprobe

✓ 2 μl 10× BAM buffer (m93)
✓ 3 μl ADA19 Ch3A DNA (≅2 μg DNA)
✓ 5 μl σ21 Probe (20,000 cpm)
✓ 8 μl d.w.
✓ 2 μl BAM H-1 (n7)

1.15 pm to 37°C – 2.30 pm



On 2.35 pm

100 mA constant current

0.75% Agarose ~4 V/cm

4× Flemings

Off 4.45 pm

Fast fried & set to expose at 5.45 pm – 8 pm

8 pm → 8 am

Resuspended in Cet Br & photo'd

O.K. to use – but clearly 15°C will be better.

Fri. Christmas Eve

Bag + ~30 ml 2×SSC/½% SDS. Drained. Reattd. Drained. Floated on & turned over in 2×SSC/½% SDS. Incubated ~½–¾ hr. Ditto at 70°C. Drained (bag sealed) & replaced. Supert was colder than filter so counts that are left on for 2nd wash are hard a. After second ≥½ hr 70° wash rinsed for a few mins. in 2×SSC & assembled on 3MM for drying. Bit bit bold together, so stapled between two papers. Rough autorad from ~3pm to 10am. through 3MM. (old film)

[See o31 for results]

Back on for more exposure without paper between ~11.30 am → 9 am
25th 27th
(new film)

12.15 pm back to hybridn. wrd. ~0.25 ml more of buffer.

Bill Singer's tips

~ 10^6 - 10^8 per sheet. (0.2 - 1 μg)
baked
Wet filter with 6 × SSC
Put in bag with 150 ml hybrid. sol^n
at 70°C ~ 12 hr (or overnight)
Full size + 3 - 5 ml hybrid. mix
Overnight or 24 hr.
Remove
Wash 1% SDS 2 × SSC
— many times every 15 mins.
for ~ 6 hr.

Took the baked gel transfer Millipore
(broken badly by vac. release !!)
transferred to 150 ml Denhardt's 6 × SSC Ficoll,
~ 5.0 pm BSA, PVP buffer & to ~ 68°C - 70°

9.30 am removed buffer & replaced with
3 ml hybrid^n (i.e. + SDS) Denhardt
+ 200 μl probe & 21 i.e. 0.8 × 10^6 c.p.m.
Mistake 1 — should have denatured
the probe first !!

11.50 am ∴ took 200 μl more of probe + 22 μl 2M NaOH
11.56 am — then plus 44 μl | 1M HCl
1M Na_2 Phos 1M NaH_2 Phos

Thurs. Dec. 23rd

Check of new DNA's → of enzymes

Following enzymes are needed:—

(R1) DGR1 is good → o1 Sugden's 10x + ATP + DTT is checked.

(ligase 10) checked extensively → its the best we have

(DNA Polymerase I) not yet checked ∫ Behring

(Bob Wells t.t.) tested well ~ n 109

(Harvey will check this)

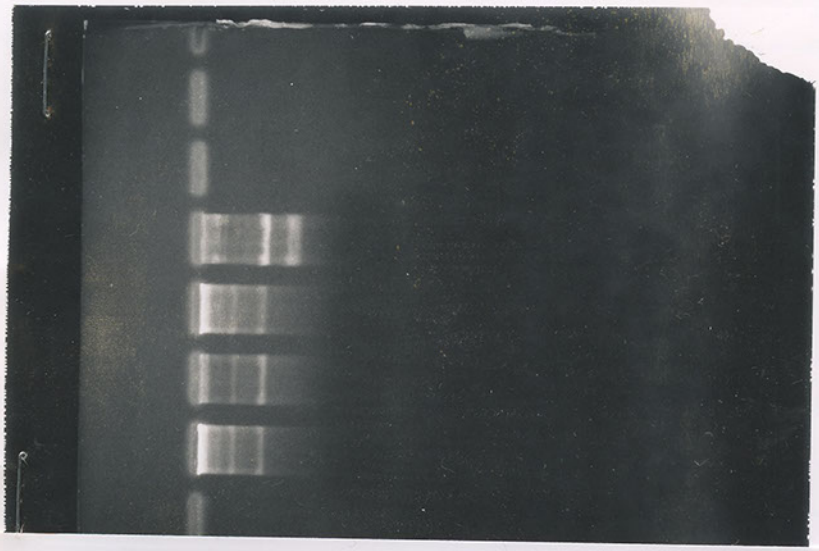
Check new DNA's with R1 / ligase ± conditions plus no enz. control

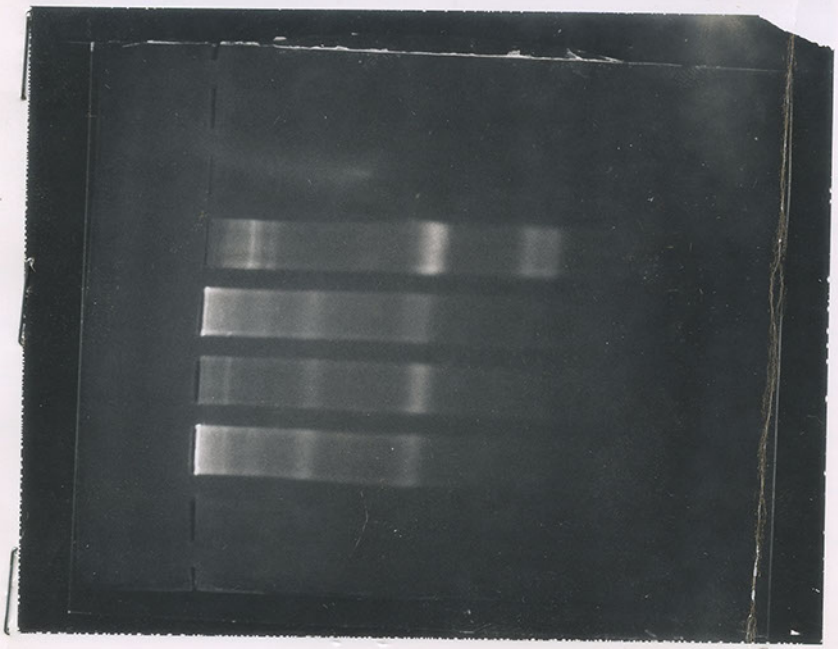
Conditions are ~~for~~ each DNA

~ 2μg DNA
2μl 10x Sugden's R1 + ATP + DTT (o1) + 9μl d.w + 1μl
2μl ligase 10 / 6 [2 ligase 10 [illegible] 10x R1]
2μl DGR1 / 50 (n92)
Water to 20μl

ligase 10/6 9µl d.w. 1µl 10xR1+(o1)
2µl ligase 10

028 DGR1/50 DGR1 1µl EcoR1 preserving buffer 50µl





Fri. Dec 24th

DNA ~~tests~~

3A - lac	1.35 μg/μl	– take 2
Hb 4	1.075 μg/μl	– take 2
Hb 9	350 μg/μl	– take 6
Hb 11	425 μg/μl	– take 5

– these were made on the 20th & so have not had v. long to sticky end in the cold.

	3A-lac	Hb4	Hb 9	Hb 11
10xR1	2	2	2	2
DNA	2	2	6	5
d.w.	12	12	8	9
ligase 10/6	2	2	2	2
DGR1/50	2	2	2	2

To 37° at 10.30am

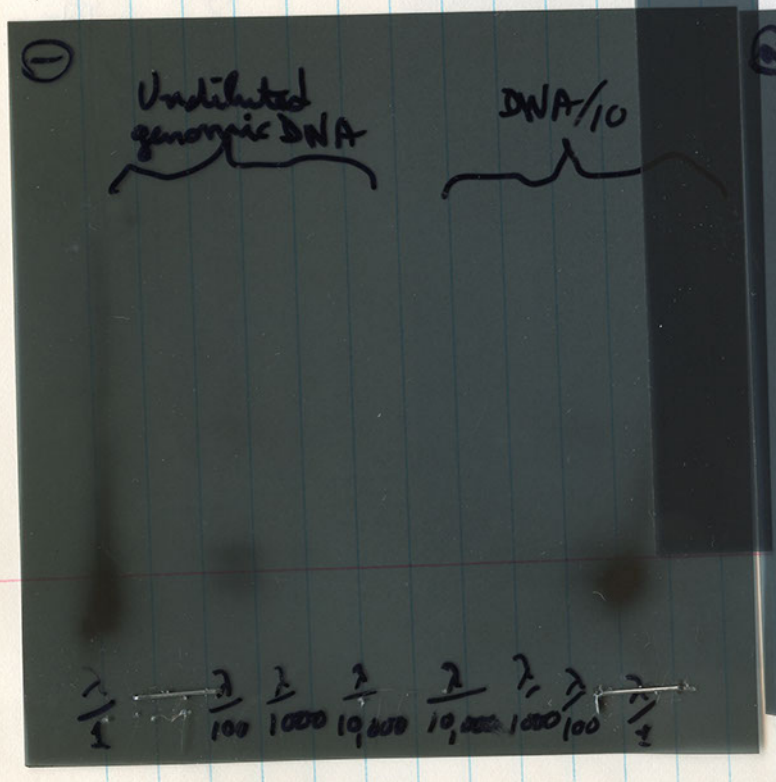
11.40am to ice

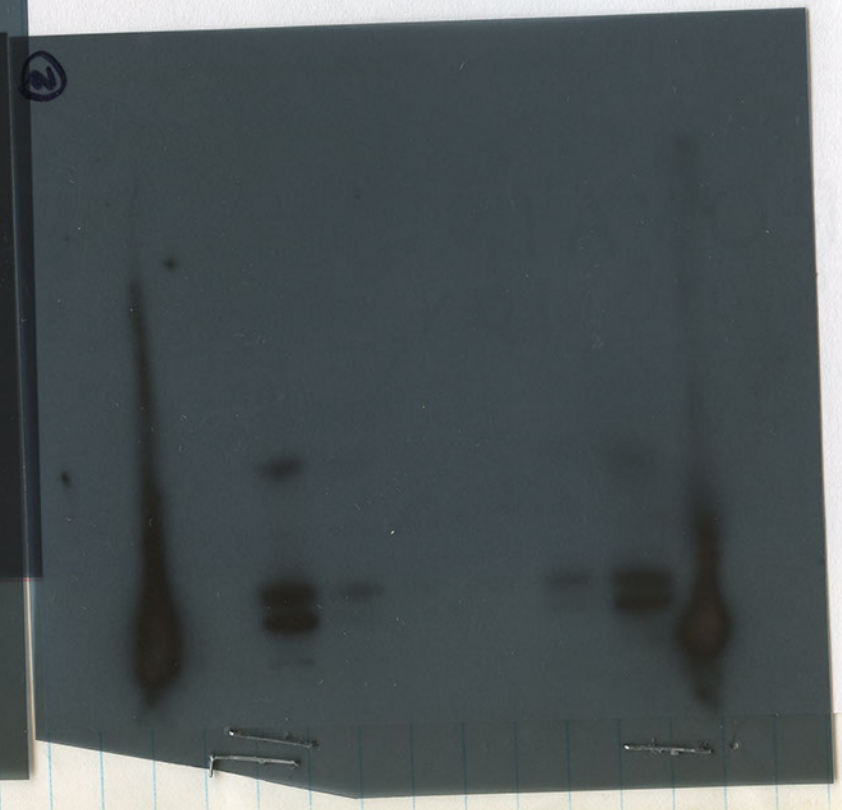
Stopped at 11.50am with 2μl 3M NaOH made up in 1M EDTA

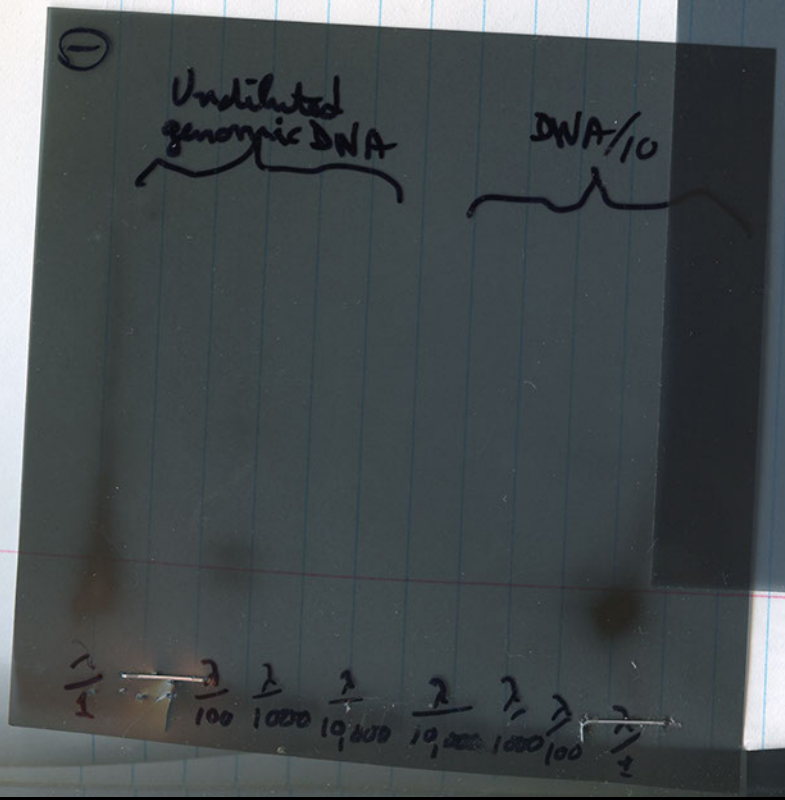
On 12 noon Off 2.30pm

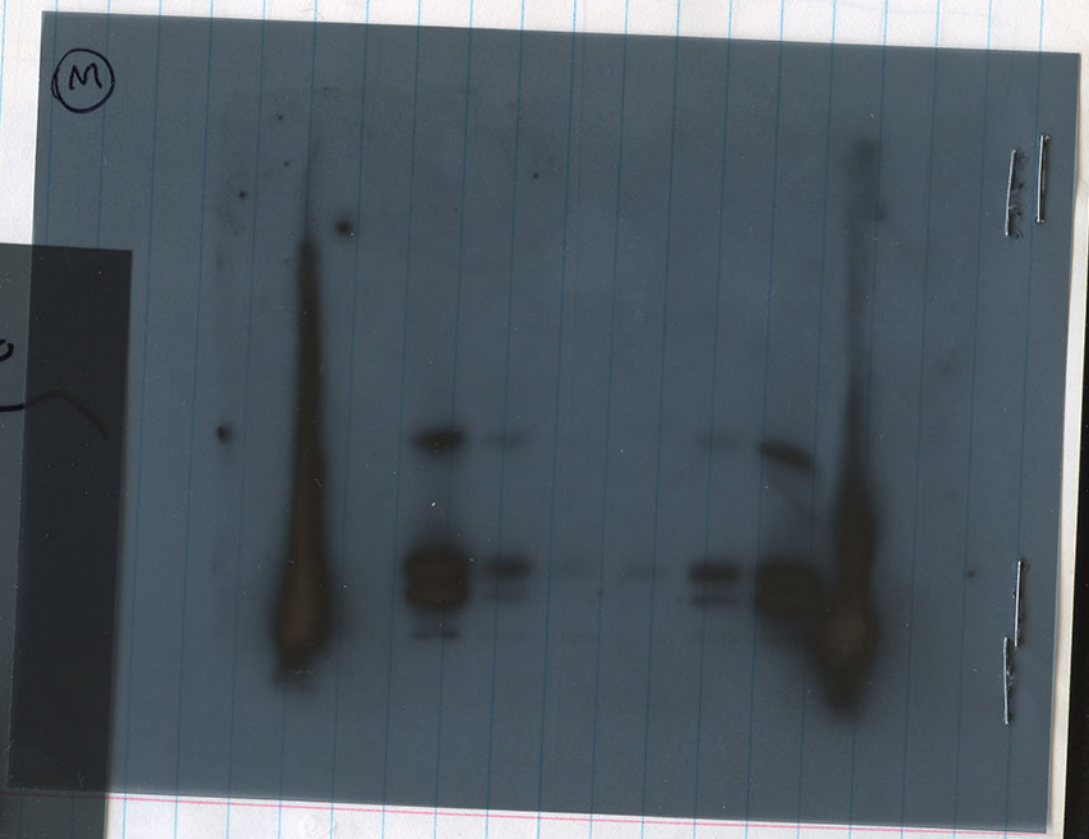
3A-lac	0.75% Agarose	Re soaked in H×4 & started up again 3.30pm at ½V/cm
Hb 4	H×4	
Hb 9	3V/cm	
Hb 11		

Conclusions: ligation is good in the 3 Hb clones & fragments in 3A-lac. NcoR1 ~~[illegible]~~ sites on Hb


(−)
Undiluted genomic DNA
DNA/10
λ/1
λ/100
λ/1000
λ/10,000
λ/10,000
λ/1000
λ/100
λ/1


(M)
(N)


Undiluted genomic DNA
DNA/10
λ/1
λ/100
λ/1000
λ/10,000
λ/10,000
λ/1000
λ/100
λ/1


M

Sat. Dec. 25th (Christmas Day!)

Transfer text

Results show even on first try, reasonably good transfer & sensitivity. No background — so very prolonged washing is not needed.
Total washes were i) overnight prewash
ii) Several rather crude rinses with $\frac{1}{2}$% SDS 2xSSC
iii) Transfer to floating on Sello
iv) One ~$\frac{3}{4}$ hr 70°C wash — probably this one was all that was needed
v) Second ~ 1 hr 70°C wash Sello
vi) Brief 2xSSC room temp wash.

However it is clear that the probes need to be at least 1 order of magnitude better, or more DNA. Can just see 1 copy on 10^{-4}
Did not overload gel with ~ 4μg in a slot but this was only ~ 0.3μg/μl & can expect to go to 5μg.

∴ on next test use ppt'd. R1 DNA at higher conc^n.

Fri 3pm → (1) ✓ use ppt'd. R1 DNA at higher conc^n.
Exposed Sat am. → Maram. (3) → Maram
New film (2)

Mon. Dec. 27th

Euk DNA tests

In view of λ hybrid. Southern test success decided to try mouse DNA for cDNA probe. Isolate:-

~5g mouse liver (~5mg DNA according to Dr Mars)

Grind in liquid N_2.
Add to 50 ml { 0.5M EDTA, 2% SDS } pH 7 at 60-70°-80° (dif not exceed 80°)

Decanted to ~5 g equiv. of liver which is fairly reasonable ppt'd mess.
Held at 80° – 70° for ~30min

RAF RNA'se n.13 (reheated) added to 10μg/ml
i.e. 500μl of 1μg/μl
– allowed to cool to ~60°C
& added PRONASE m 141 to 50μg/ml i.e.
~12.40pm 500μl of 5μg/μl – no effect – must be too low (it is 4x less than I used previously)
Shd. try 20 μg/μl So added to 50 ml 1-2 mg solid
1.10pm
– still no sign of activity. Put to 56° bath.
– no sign of digestion.

Try Stafford's conditions.

Stafford's procedure: –

Make 0.5% Sarkosyl in 0.05M EDTA pH8
Added Proteinase K to 100 μg/ml. (5mg to 50ml)
Heated to 50°C & added powder
slowly. Temp. fluctuated rather a lot
& once to 60°C – but all was added & went
very viscous. ≥5g K & 50ml. Added ~1mg extra proteinase
left from 4.0pm to ~4.30pm at
~50°. Still cloudy & η reduced a lot – but
there will be glycogen present etc!
Better procedure is probably a compromise
– {0.1M EDTA pH 8 / Sarkosyl} – 70°C until well
dispersed. ↓ to 50°C for proteinase K
addition & digestion.

By asm. the proteinase K looked
good. Prenase didn't go & wouldn't
get viscous even with sarkosyl
– abandon.

Tues. Dec. 28th

DNA (cont'd)

Cent.d Stafford mix on IEC to remove (small) amount of undigested. Ferritin? or glycogen? Did not come down. η still same as yesterday.
Extracted ~~once~~ 3X with aq. sat.d (0.1M Tris pH 8 or adjusted w. Tris solid) then 0.1% HO Quinoline phenol. No viscosity. Try again.

~5.5g powdered & to 0.1M EDTA pH 8 mag stirred (max.m) with 0.5% sarkosyl at 65°–25°C
Took ~20 min. to add all powder to 50 ml.
Held 15 min at ~70° – It ~~[illegible]~~ curdy & ppt.
not the goocy mass of first stage. I suspect too low sarkosyl. Temp. to 55°C then added 5 mg proteinase K.

It was! I added 0.5% of 30% sol.n ∴ readded. η immediately began to ↑. Repeat at 50° with 1% sarkosyl & with enzyme.

Trial 3

0.1M EDTA pH 8
1% sarkosyl (made from 30%) i.e. 3.3%
Proteinase K 100 µg/ml
Temp 50°-55°C
Sloo powder affinity.
max. mag. stir
Very much better (strings up to 4 feet)

2.35
|
3.05 Then to 55° incubation 3.15pm – 4.0pm

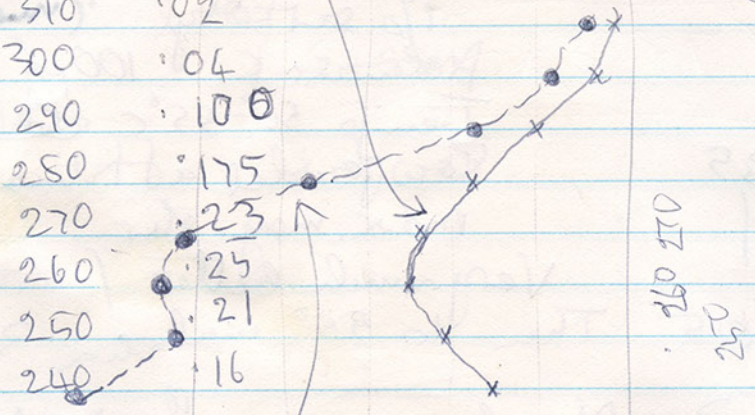
3 x Phenol (viscous!) pouring off DNA (extremely viscous!) Cent'd. & left with left phenol in the cold. Decanted & recent'd.

DNA

Post dialysis

O.D 10 µl of dialysed DNA in 1000 µl of dialysate

310	·02
300	·04
290	·100
280	·175
270	·23
260	·25
250	·21
240	·16



Conc^n $0.25 \times 50 \times 100$ µg/ml

or 1.25 mg/ml

but this will be over estimate as dialysis was not extensive

Predialysis

OD 10 µl in 1000 µl

310	·04
300	·08
290	·17
280	·38
270	·51
260	·54
250	·52
240	·67

0.41 Mouse DNA dialysed

Wed Dec 29 ck

DNA (contd.)

24:1 $CHCl_3$ / isobutanol ~ equal vol. ext.^n gave an interface in which some objectionable pink was removed but no DNA. Decanting was again easy (uphill still!) Each time more came down. ? cent.^d time On last (3rd) ext.^n cent.^d 10,000 rpm on Sorvall for 15 min. No improvement.

pH on paper at this point is 9.0 — water this → keep phenol at 7.5 in future.

Vacced off $CHCl_3$ / isobutylalc. without problem.

~ 40 ml - 50 ml DNA ∴ added 0.5 ml ~13 RNA'se (1000 μg/ml) to give ~10 μg/ml. ($\frac{1}{10}$th of Stafford)

10.30am to 37°

12.30pm Phenol extracted 2 X more
$CHCl_3$ / isobutanol 3 X
Vac.^d [→ then must shear or R1. Need a check on this]
Recovered 40 ml. Added 400 μl 10% DEPC → to cold as

$\boxed{\text{0.41 Mouse DNA}}$

The length of string has dropped substantially since RNA'se — now ~2 inches.

4.30pm ~5 ml m to dialyse in cold v. 4 l. ~DNA buffer

32 850 cpm / 10 µl

∴ 3,285 / µl 0.43 ^{32}P λ

Total 3×10^6 (~10%)

Thurs Dec 30th

100 µl ~~dial.d from 10.25 am~~ →
~~against DNA buffer~~ .01 M NaCl
.01 M Tris } pH 8.0
.001 M EDTA

Recovered ~~[illegible]~~ ~ none (~~got out off ring~~)
~~→ blew down to~~ ~~scrapped~~ – except for use as λ control on 0.45

200 µl + 600 µl EtOH → cooled (salt comes out) – back up + 20 µl d.w.
Dialyzed v. d.w. from 11.25 am to 11.55 pm
Recovered 17 µl. at 23,000 cpm / µl

2 µl 45,800 cpm in Brayf

Future probes – make more concd. – also dialyse to 10^{-3} M EDTA alone with smaller column & fractions

0.43 (^{32}P) λ Concd.

Wed. Dec. 29th

New nick translated probe

Need good probe for assaying RI completion of enk. DNA.

Dec. 8th 1 μCi / μl ∴ now 3.5 μl × 2.5 / 2.5 μCi
~ 10 μl

∴ 10 μl of each dNTP dried down
(i.e. 4 × 0.35 × 10 or 14 μCi total)

H # 9-133 10 × nick trans. buffer 100 μl ✓
+ 900 μl d.w. + 0.7 μl ME ✓

Do in ten μl next time

100 μl ✓ ✓
1 μl Chason 3A (ADA 19) ~ 0.7 μg
2 μl m151 DNA polymerase

11.50 am To 15°C ± 2°C
12.35 pm DEPC to 0.1%
0.3 M NaCl 0.01 M Tris 0.001 M EDTA pH 7
Collected in ml & then DEPC to 0.1% again

3	<.02 mR/hr	6	15		9	2
4	<.02	7	35			
5	0.55	8	7			

Vol 920 μl + 10 μl 10% DEPC

32P
1.45 gain
50-1000 Windows

Thurs. Dec. 30th

Eco R1 digestion tests on mouse DNA

DNA is 1.25 μg/μl (still ¼ of max^m permissible) — but check: —

Dialysed control (no enz.) + 1/20
Und^d. test (enz + λ)
Dialysed bulk (enz. but no ^{32}P λ)
Dialysed test (enz. + λ).

✓ 1 ml 041 (dialysed) mouse DNA
✓ 110 μl 10x R1 Sugden's buffer

35 μl DGR1

✓ 1 μl ADA19 DNA
12 μl d.w. ✓
2 μl 10xR1 ✓
✓ 2 μl 043 (^{32}P) (concentrated)
2 μl DGR1/50 (028)
λ digest R1

5 μl ✓
+
45 μl DNA buffer ✓
~25 μl ✓
Control
Control Mouse

15 μl ✓
to
20 μl ✓
043 (^{32}P) λ (Conc^d.)
Control M/20
R1 λ + Mouse R1

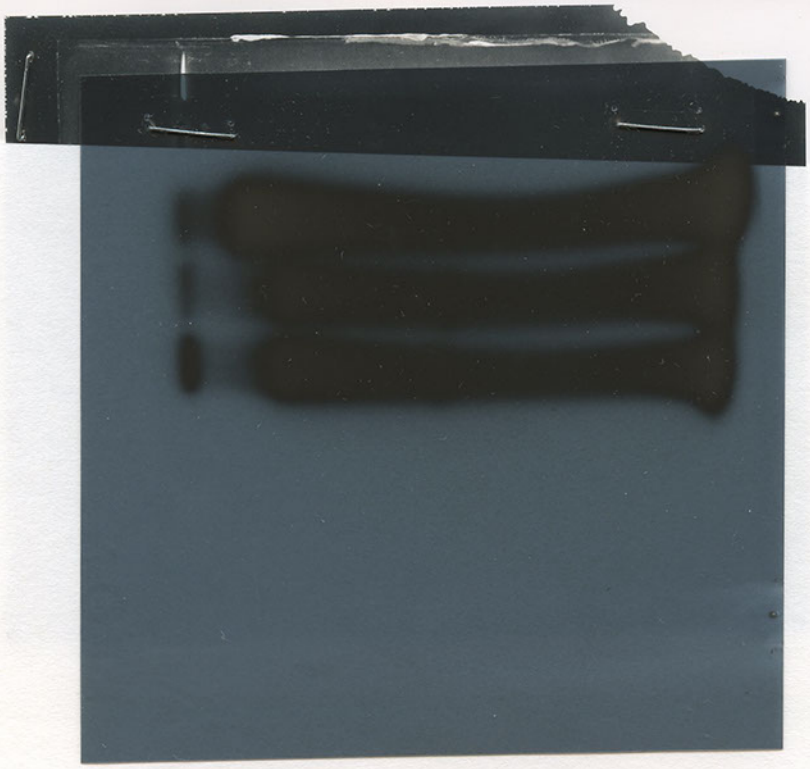
Bulk digest
045 BULK Mouse R1 Digest
Mouse R1

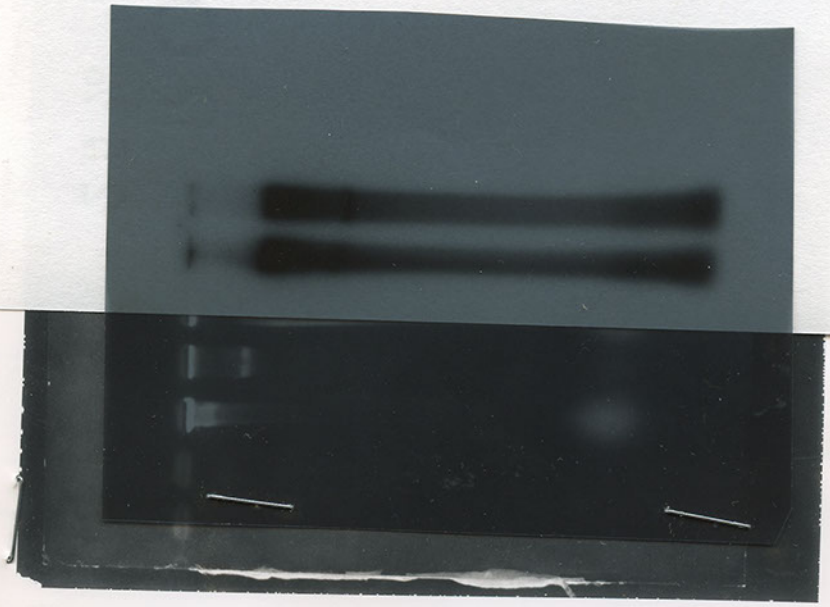
Stopped with 10 μl of 10% DEPC
λ control from 043

to 37°
1150 pm

DNA

16





On 2.10 pm off. Early am

λ control (0.43) (^{32}P) 0.75% agarose

λR1 Digest (^{32}P) 46,000 cpm H×4

{Mouse DNA + λ(^{32}P)} R1 digest 46,000 cpm 1 V/cm.

Mouse bulk R1 digest

Control mouse DNA (no enz.) /20

Control mouse DNA (no enz.) 1× (exposed Mon Jan 3 9am →

On 2.05 pm off 4.35 pm

Smaller slots 0.75% agarose

λ R1 digest ^{32}P (residues) H×4

[Mouse DNA + λ] R1 digest ^{32}P

Bulk Mouse DNA 4 V/cm

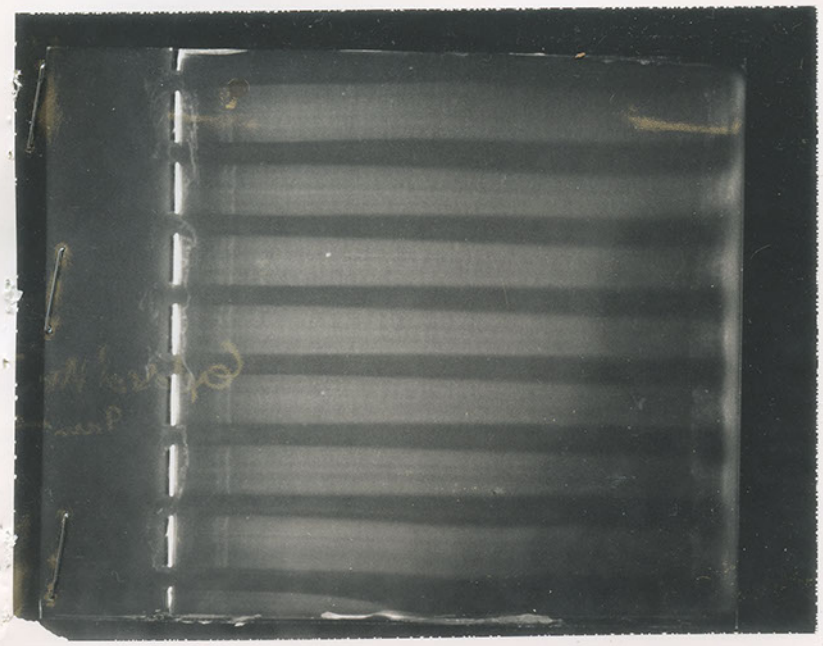
Control Mouse /20 no enz

Control Mouse /1× no enz

Brief stain.
Fast dry.
Autorad. ~6 pm ↓ ~8 am

Conclusion:

Digestion goes ok. There are quite a lot of RNA fragments in the material so DNA conc^n is probably closer to 0.5 pg/μl. The starting DNA looks ok.



3pm ~10 min in 2×SSC 3.25 fixed at 80° under vac.

~5.30pm

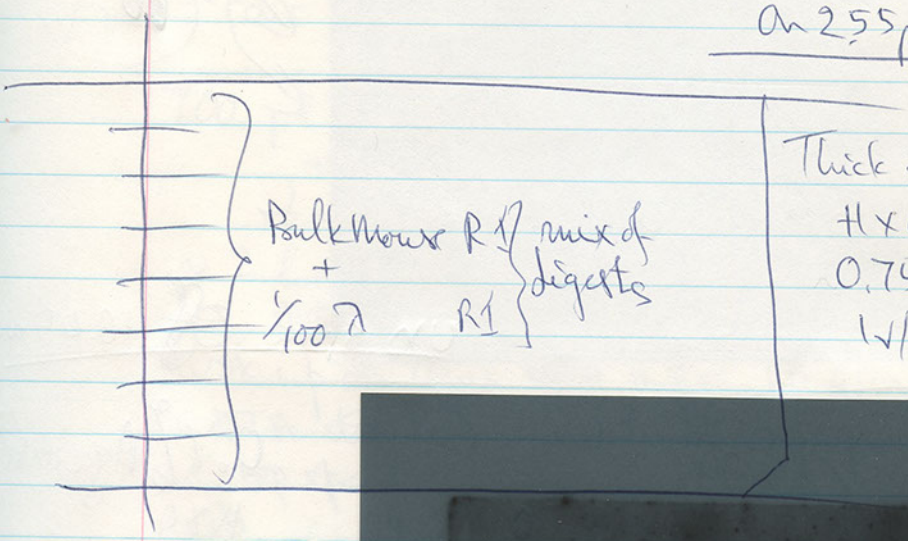
3hr. is not enough. Use overnight

Thurs. Dec. 30th

DNA transfer tests (mouse)

o45 {Bulk Digest Mouse DNA} 200 µl + 2 µl {o B λ R1 digest}

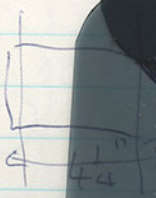
On 2.55 pm Off 9.30 am



Thick gel (25 µl slots)
H x 4
0.75% Agarose
1 v/cm

After transfer hybridize to both probes. Great control needed for fully hybridization — but in future can add native λ + see get control for digestion also.

10.30 → 10.55 Solution I
11.0 → 11.25 Solution II



12 noon 2 x 1 lb o a g

Conclusion looks like a possible positive

Allowing 30% for DNA content

20 µl of ϕ51 10x R1 Mouse ≅ 200 µg

of 100%

60 µg

Fresh 0.7% Agarose made

On 1.50 pm Off 10.45 am

Use for gDNA:
- 1/100 ϕ13λR1
- ϕ51 10x R1 Mouse (20 µl)
- ϕ51 5x R1 Mouse (20 µl)
- ϕ45 R1 Mouse (20 µl)

Use for λ DNA:
- ϕ45 R1 Mouse (20 µl + 1 µl of 100 ϕ13λR1)
- ϕ51 10x R1 Mouse (20 µl + 1 µl of 1/100 ϕ13λR1)
- 1/100 ϕ13λR1

0.7% Agarose
4x4
0.5 v/cm

Conclusion: This 10x load is a little too much

Fri. Dec. 31st

New Year's Eve

Mouse R1 transfer.
Gels show no problem with over load. ∴ ↑ conc^n.

700 µl o 45 Bulk Mouse R1 + 80 µl 3M NaAc pH 8 o to -20°C
+2.4 ml ethyl alcohol

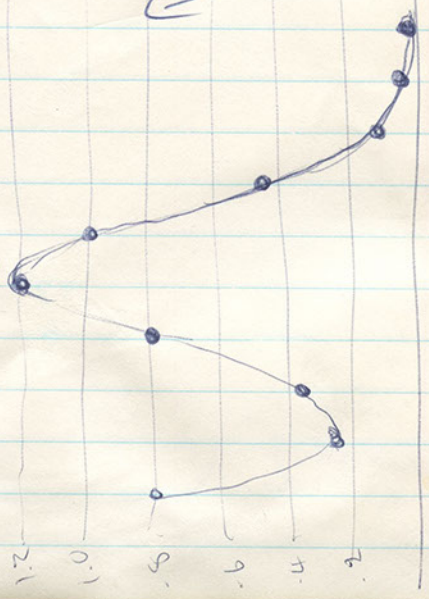
~ 2 hrs. later cent^d off at serval top.
After scanting, o Q tip wiping, took up with 70 µl buffer (0.01M Tris 0.001M EDTA sterile). Cent^d off glycogen successfully at top speed.

O.D. 2 µl in 1000 µl DNA buffer

O.D alcohol super^t 10 µl in 1000 µl DNA buffer OD < .02

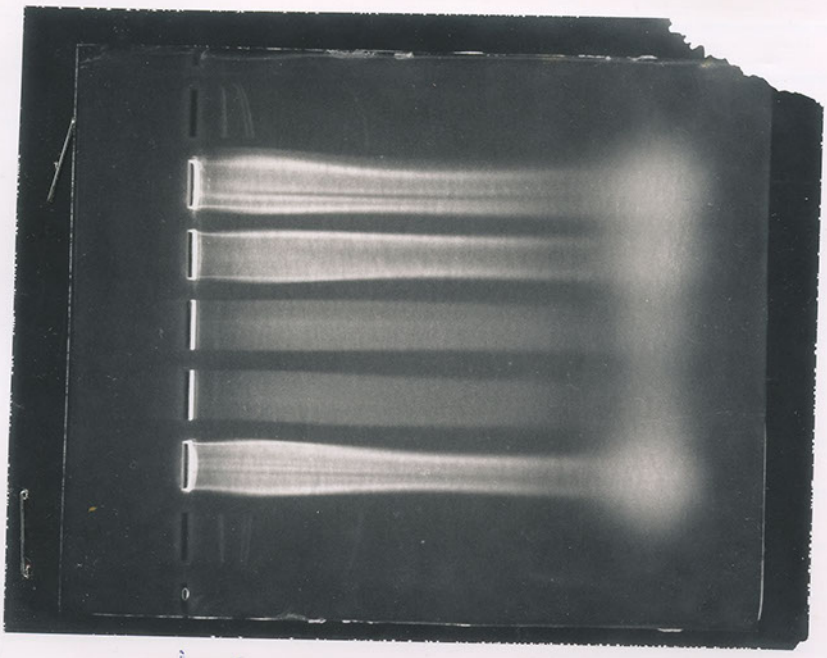
310	.025
300	.045
290	.125
280	.45
270	1.0
260	1.2 (264 max)
250	.79
240	.35
230	.25
220	.80

This O.D. is almost 2 × expected value!

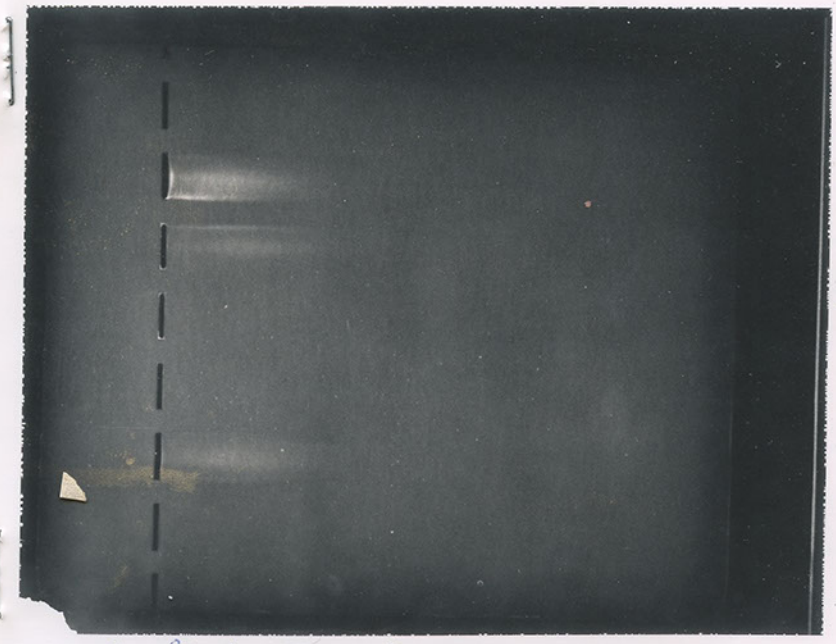


φ51 10X
R1 Mouse

DNA



looks as if 10X is overbraded.



V. effective transfer

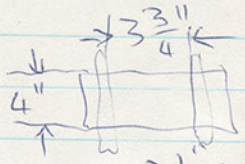
Sat. Jan 1st 1977

DNA transfer

10.50am 11.10 gel stained

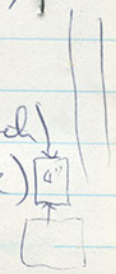
11.15am → 11.45am Solⁿ I { 1.5M NaCl, 0.5M NaOH

11.45am → 12.15pm Solⁿ II { 3M NaCl, 0.5M Tris pH 7



Glass Plate

Bottom 3MM	3¼" × 12"	(i.e. slide width)
Top 3MM	3¼ × 4"	(i.e. slide size)
Millipore	4¼" × 4¼"	
Paper towels	4" × Width	

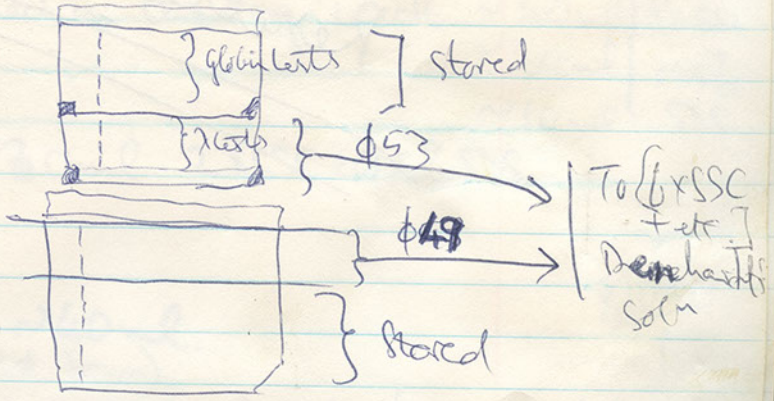


Set up by 12.25pm 2 lbs.

11.0am off → to 2×SSC. [All 20× SSC was through].

11.30am to 80° under vac (plus older φ49 test reheat)

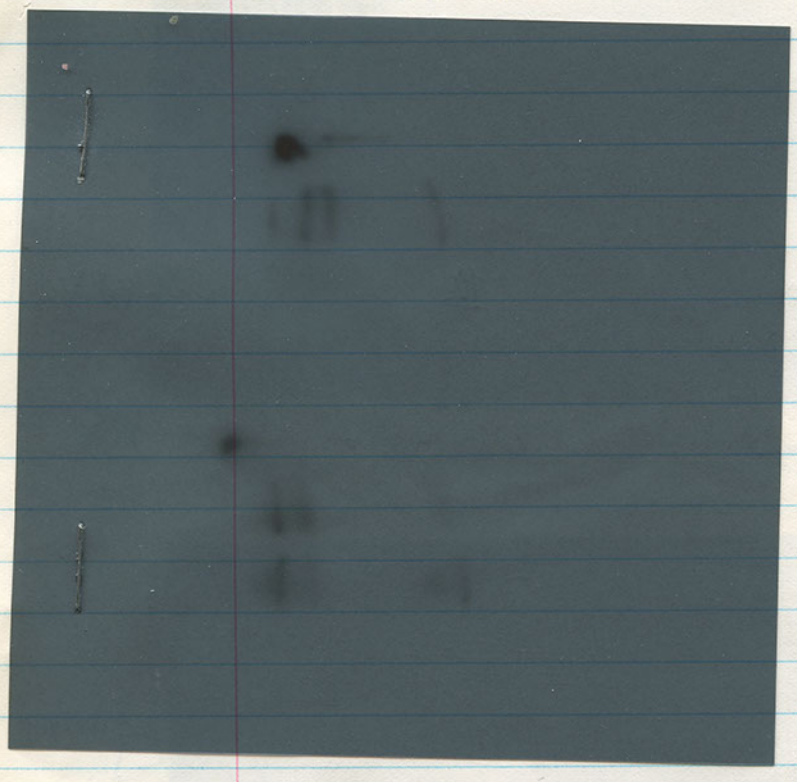
2.0pm off Cut



Also from φ49

2.30pm to 70°C 9.15am

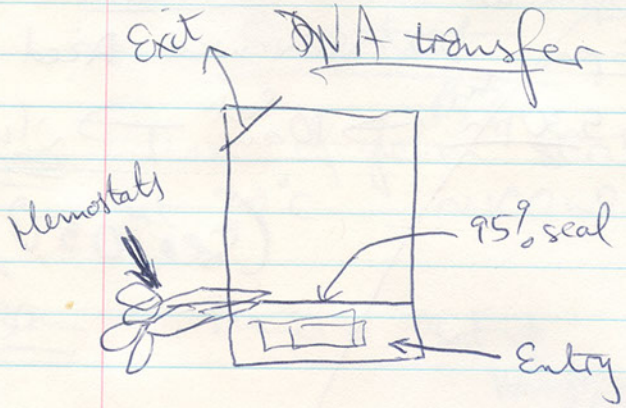
Result shows excellent transfer & successful hybrid?. E.p. is good on the 1X mouse R1 but is doubtful on the 10X. Too early to tell – but proceed with others



Results back with gels

Mon. Jan 3rd

DNA transfer



Expelled wash & sealed 95%. Closed off with hemostats

ϕ53 pieces (FN) + ϕA 9

9.48am 100 µl ϕ43 (^{32}P) λ probe (300,000 cpm) + 15 µl 2M NaOH

9.58am plus 30 µl ϕ25 neutralizing buffer { 1M HCl final, 2M Phos pH ~ 8

Then plus 2 ml of ϕ17 hybridization buffer &

Transferred to bag. Too ~~little~~ vol. to separate papers – would be okay with one.
Added 1 ml more & tried again. Not good but left – since this is a trial.

10.30am to 70°

9.0am off. Washed in the bag with ~30 ml, ~30 ml, ~10 ml { 2×SSC, 0.5% SDS

10.15am to 70° with ~30 ml 2×SSC + 0.5% SDS

↓

11.45am

12.0pm to 70° with ~40 ml. (temp. a bit down)

↓

2.30pm Rinsed in 2×SSC. Dried & to autorad 3.10pm

8.10am

Wed 8.30am → Sat 10 → N week

3.45pm Fri. Took off. Washed 2x 2xSSC+½%
& set up 3.45pm at 70°C. Second wash
4.15pm — 5.15pm 2xSSC rinsed. Dried
& to autorad. 5.30pm Fri → 10am Sat → 1 week

(see 050)

Thurs. Jan 4th

Real test

12 noon — Rest of globin strip (φ53) to Denhardt's at 70°C ~100 ml.

↓

8.30am — Drained & set up

Hemostat with rubber cover.

1.8 ml of (^{32}P) cDNA (ss) globin from Haring HF 10-54 2.2×10^6 in 0.001 M EDTA (see φ27)

∴ make up 50 ml } 12×SSC + 1% SDS + Ficoll + BSA + PVP
·02% ·02% ·02%

2×Hybrid^n buffer 12×SSC φ57 } 17.5 ml d.w.
30 ml 20×, 20mg F, 20mg BSA, 20mg PVP
Sterilized & added 2.5 ml 20% SDS

10.0am — Probe + equal vol 2×Hyb^n buffer & set up at 70°

9.30am — Wed. off — extruded hybrid^n mix up — sealed & re-used for φ49 piece

~9.15am — φ49 piece to 6×SSC Denhardt's at 70°. Wash for 1 hr only. ~10.15am to old hybrid^n from 6 & to 70°
rough. Technique not good

Rinsed strip 2× in 2×SSC ½% SDS (dropped it once!)
10am to 70°C for 1st wash (it has a hot spot) 10.45am 2nd wash
~2.0pm Off & tried after 2×SSC rinses. Autorad. 2.10pm → 10am

DNA

OLIVER

December 1976
Memo NAR-64

Specific excision of the inserted DNA segment from hybrid plasmids constructed by the poly(dA)-poly(dT) method.

H. Hofstetter, A. Schamböck, J. van den Berg and C. Weissmann,
Institut für Molekularbiologie I, Universität Zürich,
8093 Zürich, Switzerland.

NOV 1 RECD

Under conditions designed to give selective denaturation of DNA, endonuclease S1 specifically and efficiently excises a DNA insert from a hybrid at the poly(dA)-poly(dT) junctions. The hybrid plasmid PBG (Maniatis et al., Cell 8, 163), 200 µg/ml, was digested in 45% w/v formamide, 0.2 M NaCl, 1 mM $ZnSO_4$, 30 mM Na acetate (pH 4.5) with 600 units/ml S1 endonuclease (Wiegand et al., J. Biol. Chem. 250, 8848) for 30 min at 50°. This treatment cleaved the DNA into only two fragments. The smaller one, with an average of 575 base pairs, was somewhat heterogeneous and contained all of the globin specific sequences.

Since S1 endonuclease is rapidly inactivated under the incubation conditions described above, the concentration required for optimal cleavage (about 10 times that which would be required in the absence of formamide) must be determined experimentally. Underdigestion results in a somewhat larger, more heterogeneous product, while overdigestion reduces the size of the globin DNA fragment and causes an additional cleavage in the larger DNA piece.

Several viral DNAs, in particular λ, Ø29 and T7 DNA gave defined and characteristic cleavage patterns upon S1 endonuclease digestion at optimized formamide concentrations.

(Bioch. Biophys. Acta, 1976, in press)

December 1976
Memo NAR-65/1

Generation of new restriction sites by mutation in bacteriophage T5

John Davison and Françoise Brunel

OCT 16 RECD

MRC Unit of Virology, University of Glasgow.
Present Address: Beatson Institute for Cancer Research, Garscube Estate, Glasgow, G61.

Bacteriophage T5 is insensitive to *in vivo* restriction by the *Eco* R1 restriction system, although the DNA has six *Eco* R1 sites *in vitro*. We have isolated mutants of T5 which are sensitive to *in vivo* *Eco* R1 restriction and have genetically mapped these mutations in the First Step Transfer (FST) portion of the genome (i.e. that portion of DNA which enters the cell in the first 10 minutes after infection).

Tues. Feb. 15th

Grants all done! Meeting attended!

Probe for direct Hb clone detection

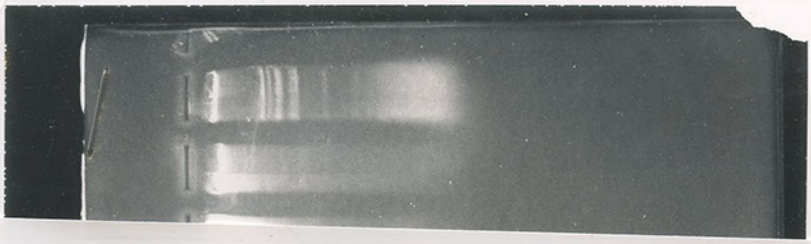
If cloned Hb is ~1% of λ then it would need 100 × cold λ to ↓ background to equality — assuming no cross-linking.) ∴ better to prepurify cloned piece prior to labeling. Since there are no R1 sites left & Hha leaves still ~5% λ DNA - need to try S1 nuclease on dA/dT tails.

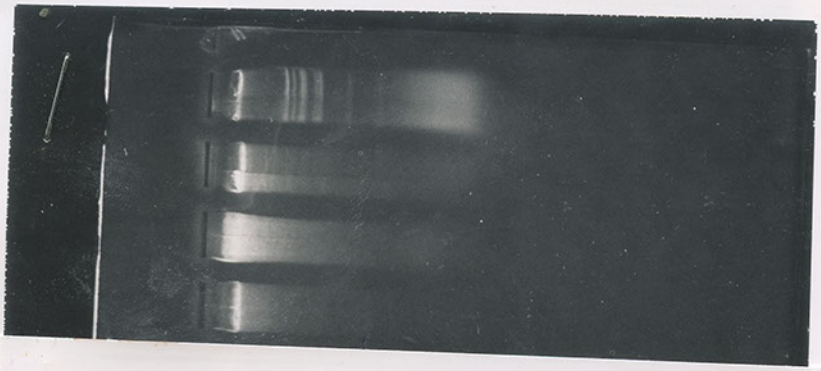
∴ try S1 / formamide on Hb clone e.g. 4
on "Hb" 3 clone (dA/dT)
on 3A Δlac

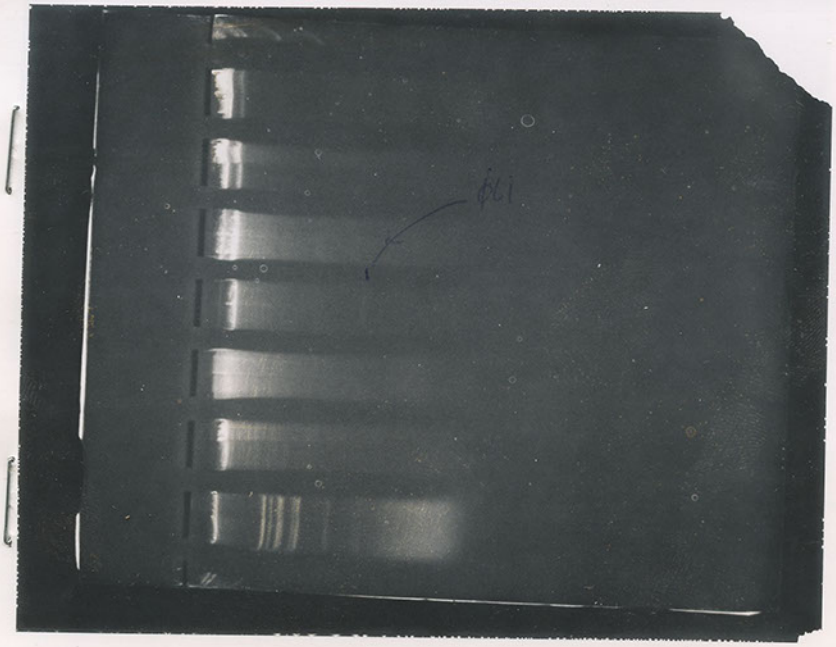
Add "stuffer DNA" ~1μg/20μl R^x mix
(Harvey has 10-113W Ch 3A Δlac at 0.66μg/μl in DNA fraction for 3A Δlac. For stuffer use "Ch 3A/R1 10-23" at ½μg/μl in d.w. Heat before use 5 min at 100°C)

10 × S1 buffer (Harvey 9-131) — filter sterilized
2M NaCl
0.5M NaAc
10 mM $ZnCl_2$
pH 4.5 with NaOH

Dilute S1 in 50% glycerol. Available is: HF 10-19 ex JR 1μl/μ
(Harvey has some) which works on 10μg DNA in 1 ml R^x






φ61

Marker is cB2 KH100 min 5

3A Hb4 OD = 3 i.e. ~ 0.15 μg/μl (label φ61)

3A Δlac 10-113W at 0.66 μg/μl

3A Hb3 at 0.65 μg/μl (m93)

Test:—

	1/10	1/3	1	3	Hb4	Hb3
Deionized formamide	9 μl	9 μl	9 μl	9 μl	9 μl	9 μl
10 x S1 buffer	2 μl	2 μl	2 μl	2 μl	2 μl	2 μl
Stuffer DNA (HF10-23)	2 μl	2 μl	2 μl	2 μl	2 μl	2 μl
D.W	3	3	3	1	—	3
Ch3A Δlac	3	3	3	3		
3A Hb4					6	
3A Hb3						3
S1 nuclease	1 of 1/10	1 of 1/3	1	3	1	1

13 μl of 63 μl + 14 μl 10X + 14 μl HF9-13

2 22pm to 50°C (air) 3.0pm to ice.

n21 Hb3 BAM

New Marker φ60 (by mistake in 20X Hennings)

3x S1 3A Δlac On 3.45 pm

Hb3 1x S1

Hb4 1x S1 1.0 V/cm

1x S1 } 3A Δlac 4X Hennings

1/3 x S1 0.7% Agarose

1/10 x S1

n21 Hb3 BAM ← φ61 Off 10 pm

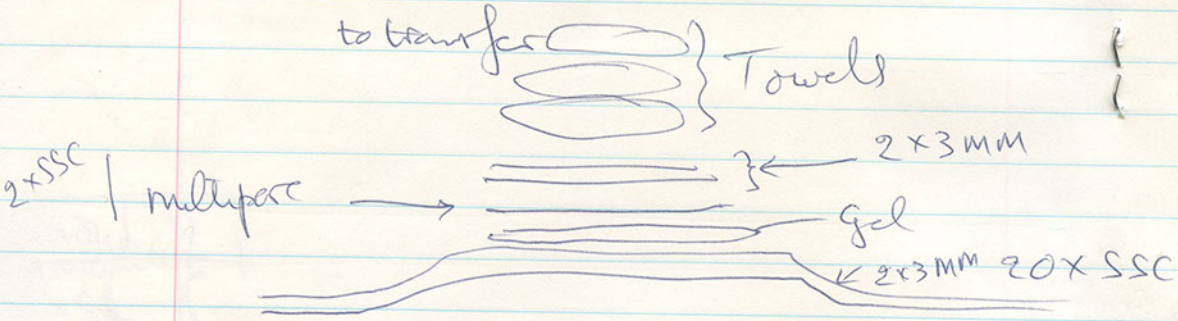
A possible faint band on Hb4 : test with probe → (over)
and repeat without "stuffer" DNA

10.40pm to sol^n. I (1.5m NaCl 0.5m NaOH)

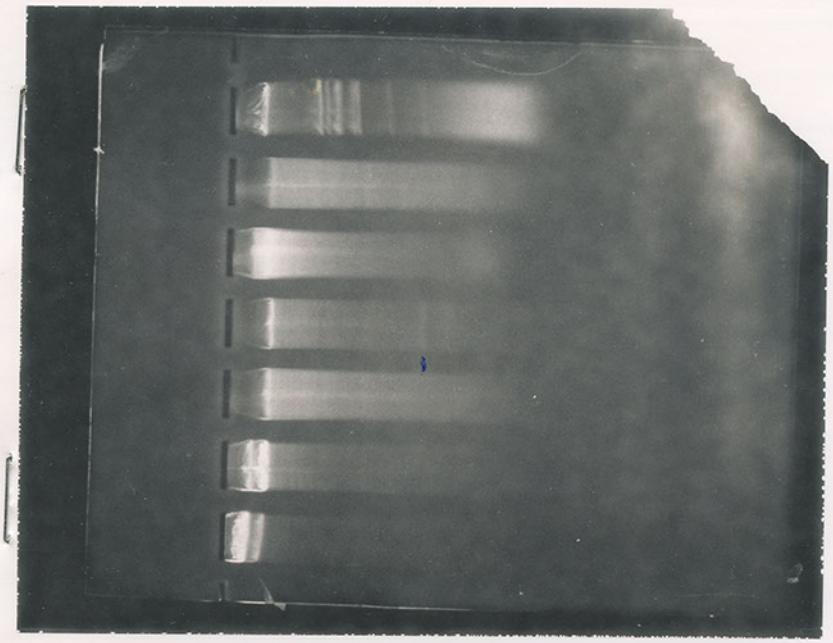
10.55pm — sol^n II (3m NaCl 0.5m Tris)

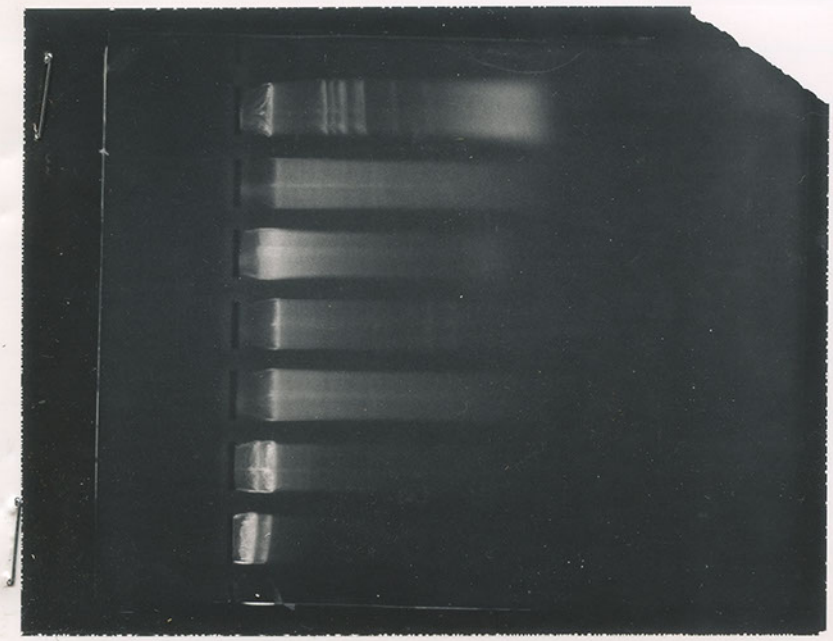
11.10pm

to transfer



11.20pm away. Next a.m. dried ~2 hr at 80° after 2xSSC rinse





Friday Feb 18th

Repeat S1 tests without "stuffer" DNA

9 a.m.

	1/10	1/3	1	3	Hb4	Hb3	
Deionized formamide 10 x S1	11µl ✓	11µl ✓	11µl ✓	11µl ✓	11µl ✓	11µl ✓	Deionized formamide 63µl ✓ + 14µl 10 x S1 buffer
P.W.	5 ✓	5 ✓	5 ✓	3 ✓	2 ✓	5 ✓	
Ch3A Δlac 10-13 W	3 ✓	3 ✓	3 ✓	3 ✓			
3A Hb4					6 ✓		
3A Hb3						3 ✓	
S1 nuclease	1 of 1/10	1 of 1/3	1	3 ✓	1 ✓	1 ✓	

2.25 pm to 50° ± 2° (trouble with thermostat)

½ hr takes to ice

On 4.45 pm 1.0 v/cm 4 x Hennings 0.7% Agarose

Off 11.45 pm

Markers mix
Δlac 3 x S1
Hb3 1 x S1
Hb4 1 x S1
Δlac 1 x S1
Δlac 1/3 x S1
Δlac 1/10 x S1

Conclusion: Same piece in Hb4

It is ~ 3.4% λ!

17/14.22 37/14.22

Saturday Feb 19th

Preparation of probe

If the φ61 piece is correct can use it to tell Hb+ from Hb− simply. Rather than prepare it & label (which saves label but is more wasteful of DNA & time), I will label it & isolate. This will give enough for test.

Suppose it is really 1% of λ, & that at 15°C I can get 10% incorp^n. Then 0.1% of total label will be in φ61. With 50μCi this will be .05μCi or 100,000 cpm. — enough for a test but it is clearly better to go the other way round finally (i.e. purify & then label)

Take 30μl of dATP
30μl of dCTP
30μl of dTTP
+ 60μl of dGTP

(2nd Feb lot. 1977 1μCi/μl) i.e. 42.3μCi total

(11th Jan 1977 1μCi/μl) i.e. 9.6μCi

52μCi total

100μl HF 9-13-3 10× nick translation buffer
+100μl d.w. + 0.7μl mE
← Note

80μl to dried down (^{32}P) dNTPs ✓
✓ 20μl (3μg) 3A Hb4 DNA ✓
✓ 2μl DNA polymerase m151

To 15°C ±2°C at 2.10pm (except for brief 20°C)

10μl of 1% DEPC at 3.10pm

To DNA buffer .01M Tris .001M EDTA pH 8.3
G100 column.

1	<0.1 mR/hr	6	38
2	<0.1	7	6
3	<0.1	8	0.9
4	0.45	9	0.4
5	38	10	~ same

Pooled 0.72 ml.

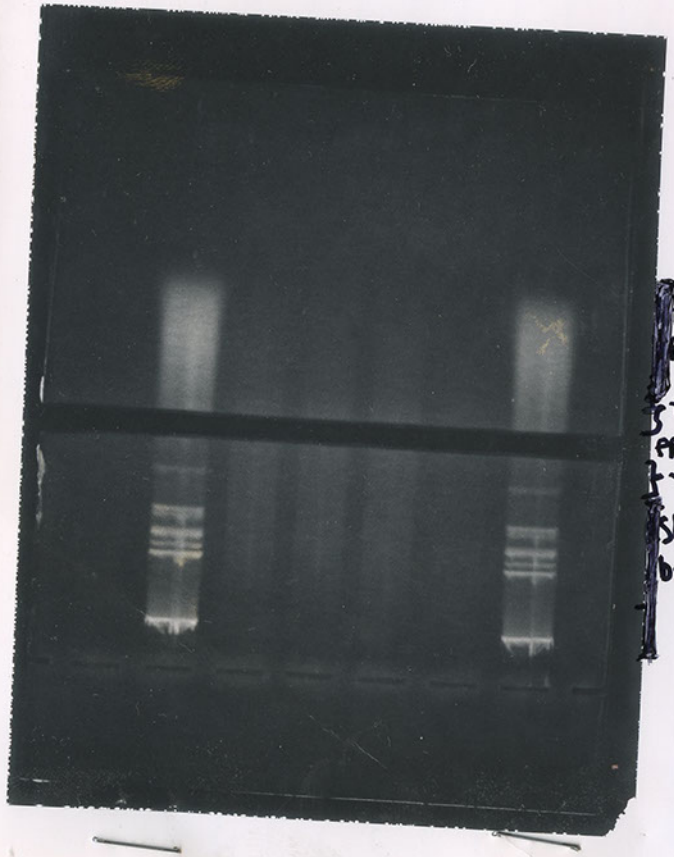
5 µl in trays gave on 1.45% gel 74300 cpm
50 — 1000
E F

Total ∴ $\frac{74300 \times 2 \times 72}{2.2 \times 10^6}$ = 4.9 µCi (close enough to 10% with unpooled #7).

Added 75 µl 3M NaAc + 2.4 ml EtOH
– cooled in dry ice & left at ~20°C from 4.10 pm to 4.35 pm. Cent! Sorvall (same losses – difficult to fill – up to 20%)
Washed 1x with 80% EtOH. Blew down.

✓ Take up in 60 µl of { 45 µl formamide, 10 µl 10x S1 buffer, 45 µl d.w. }
Then to 50°C & + 3 µl S1 HF 10–19 Ex JR at 5.25 pm

5.55 pm Froze. [069 Post S1]



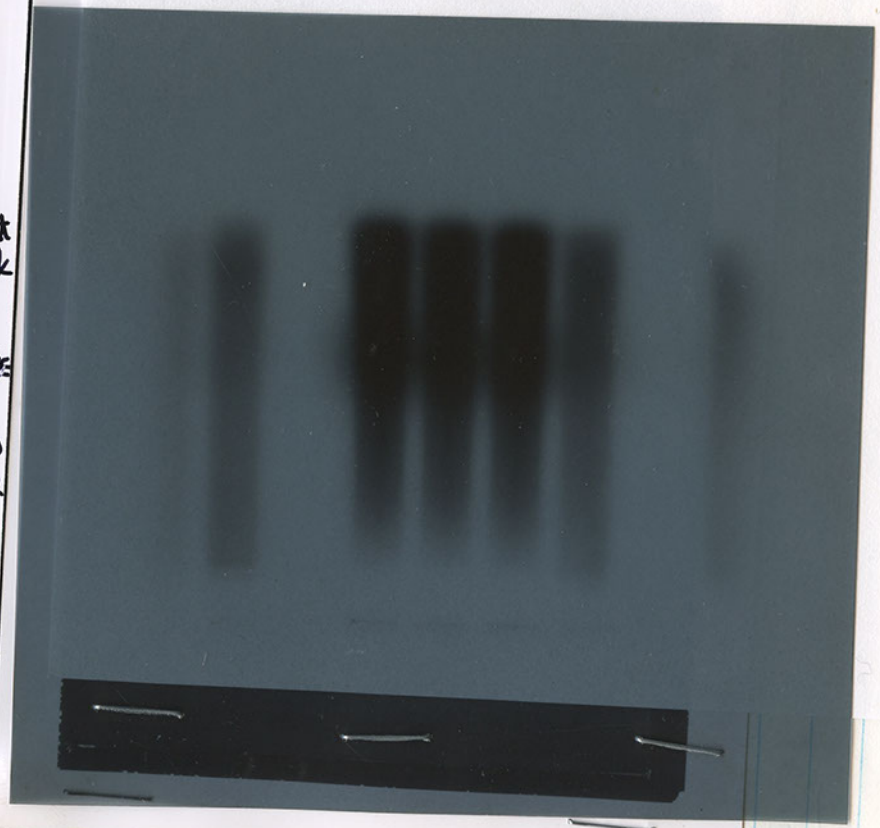
Fast bulk

+1

PROBE

-1

Slow bulk



Sun. Feb. 20th

Preparative run

On 12 noon Off 4.15 pm

Diluted probe 069 Post S1
Marker Mix
Probe 069 Post S1
3 Lots diluted
Marker Mix

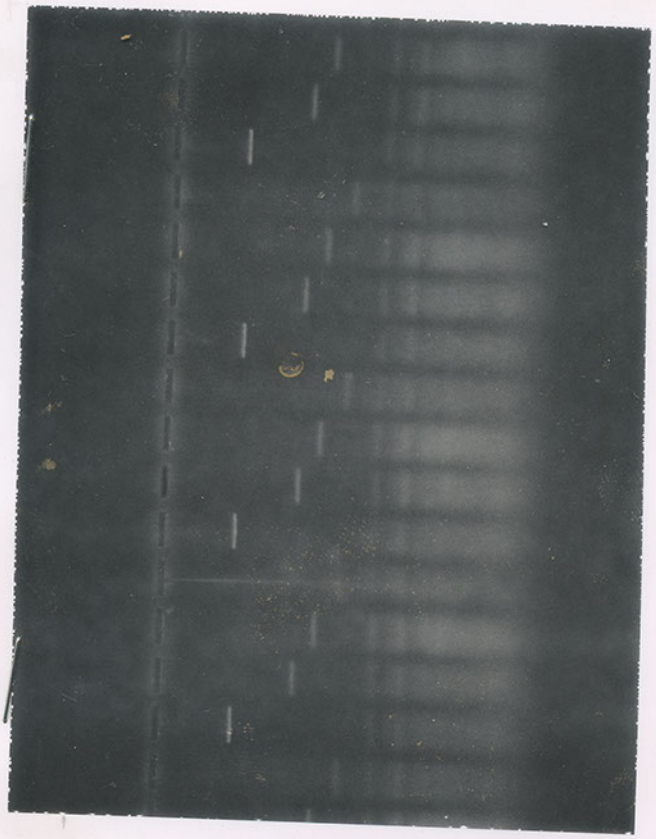
0.7% Agarose
H×4
2v/cm 67mA
Air cooled by fan

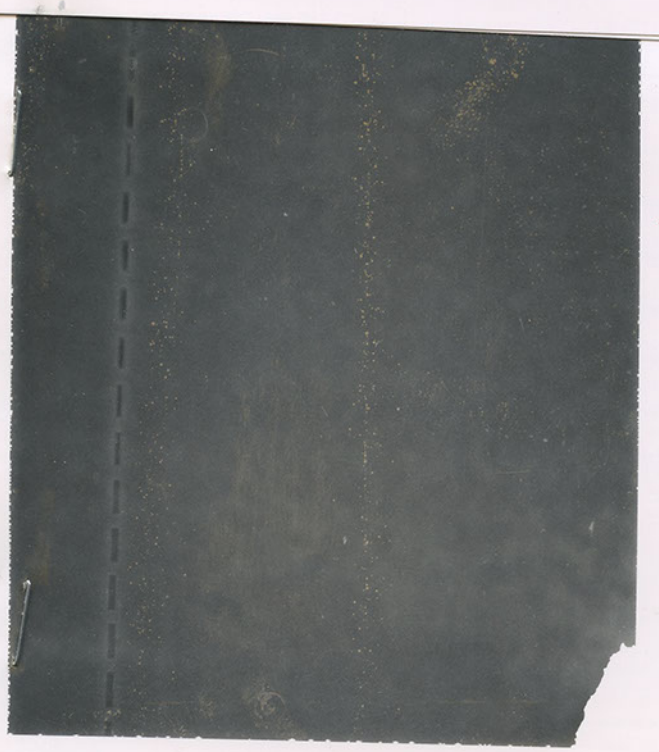
On to Saran Wrap autorad at 4.25 pm
developed at 4.55 pm for quick look
Pattern dark enough but bands just show
∴ Stained. Cut out

~8mR/h ~6mR/h
PROBE band (i.e. marker)
~10mR/h PROBE +1 and −1 (one with a head → behind)
BULK Slow → Fast (to cover autorad. area)
Frozen

~5mR/h ~16mR/h

DNA





1 Tris 0.06M Mg 0.06M Mg pH 7.9

units / µl

Monday Feb 21st

Probe test

Can test the five potential probes: —

Hha digest — transfer — hybridize

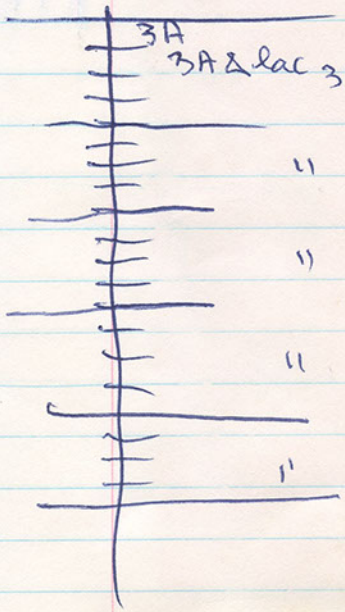
Ch3A, Ch3AΔlac, Ch3AHb3, Ch3AHb4

	3A	3AΔlac	3AHb3	3AHb4 ← (~ 2μg total)
10x Hha Buffer	7 μl	7 μl	7 μl	7 μl
ADA19 (~0.8μg/μl)	2.5 μl	—	—	—
10-113W (~0.7μg/μl)	—	3 μl	—	—
m93 Hb3 (~0.65μg/μl)	—	—	3 μl	
061 Hb4 (~0.15μg/μl)	—	—	—	13 μl
D.W.	58 μl	58 μl	58 μl	48 μl
Hha enzyme	2 μl	2 μl	2 μl	2 μl

To 37°C at 11.07 am → 1.07 pm

Ice

Gel is 95 mm × 147 mm



2x [95 × 285]

2x [95 × 147]

Milli [110 × 150]

On 1.30 pm 0.75 V/cm 55 mA

4 + H 1.5% Agarose

Off 11.30 pm soaked 15 min Sol^n I, 15 min Sol^n II

Banding KI — Sulfate Polysaccharides — Try DEAE

6M KI 10mM $NaPO_4$ pH 7

Load on Fine commercial HA BioRad

Wash with Low PO_4 to get rid of KI

KI to get rid of Dyan

Lo PO_4 → KI

Batch

100% Infectivity on SV40

Hin II or Hin III

Confidential Legato

Tues. Feb 22nd

(Transferred as usual)

2×SSC rinse
Bake ~24 80°
Overnight 4:30pm → in sterile
Denhardt's 6×SSC at 70°C with slake

Wed. Feb. 23rd

Probe extraction

Plan: Will try boiling water bath to melt Hennings 4X-containing gel (it will be torn a bit from staining). + equal vol 4XH. Then add 1M NaOH to give 0.3M followed by ~~φ~~25 2M Phos pH 7 neutralizing buffer (equal vol.). Then add 6XSSC.

Test P-1: Took out frozen probe-1. Lightly centrifuged after thawing. Recovered

0.6mR/hr 581 mg liquid } 876 mg total.
1.4mR/hr 295 mg solid }

↑ Added 0.6 ml 4X Hennings (pH 8)
Boiling water <5 min. — dissolved.
Added back liquid to ~1.5 ml total
Coded ~~[illegible] ml 2M NaOH~~ & it reset
& re melted & added 1.5 ml more 6XSSC ~~[illegible]~~
Added 0.5 ml 2M NaOH pH 12 ~~φ17~~ Annealing
∴ 0.3 ml more
Transferred to 1.6 ml neutralizing buffer
pH close to 7 & semi solid. Used all.

Test P+I

0.79 g total — ϕ17

Added 2.5 ml 6xSSC (Denhardt) Annealing buffer

Boiled (pH 8-9 - closer to 9)

Added 0.5 ml 1M NaOH pH >13

Added 1 ml neut.g buffer (use a little less)

— — back titrated to pH 7.5.

Set up.

Probe test 0.72

Added 2 ml 6xSSC Annealing

Boiled

Added 0.5 ml 2M NaOH

Added 0.8 ml neut.g buffer — pH 7

added 0.1 ml 1M NaOH — pH 7.5

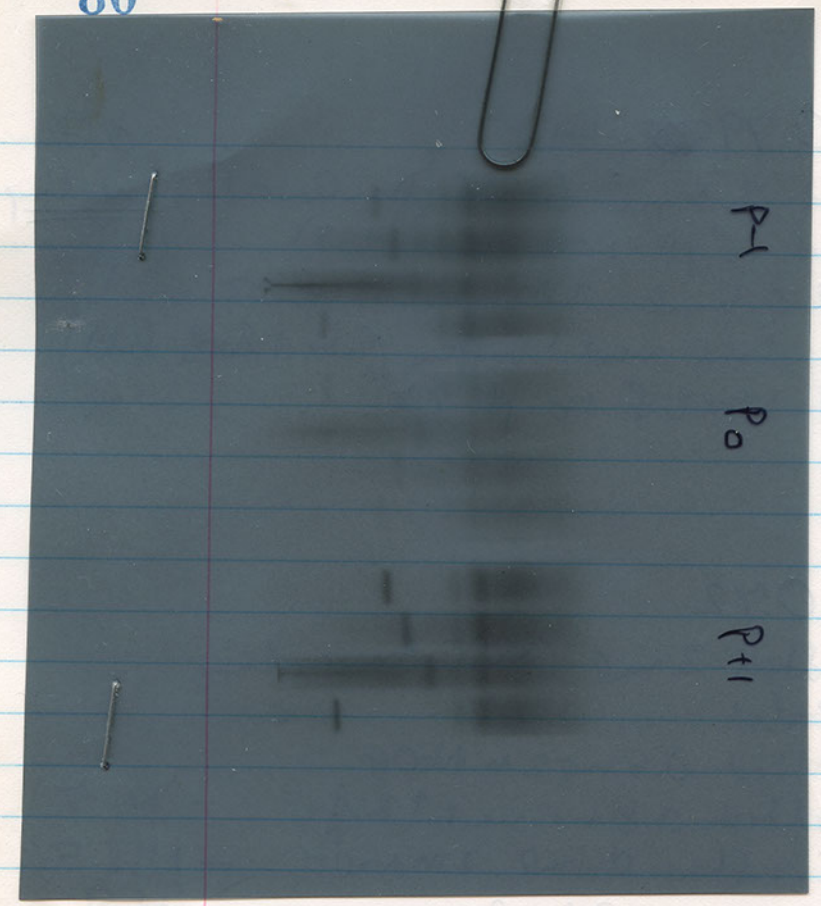
— — — 0.1 ml — — — pH 9

— — — 0.025 ml neut.g pH 8.5

Used ½ for test. Froze rest.

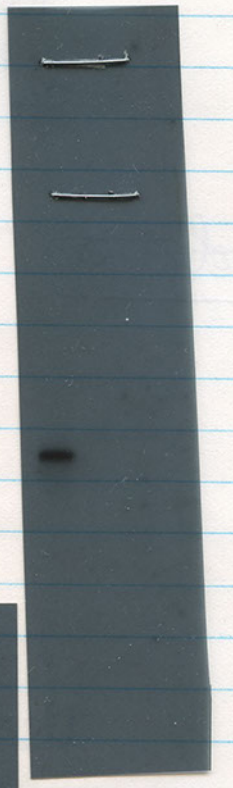
as ϕ79 P_0 PROBE

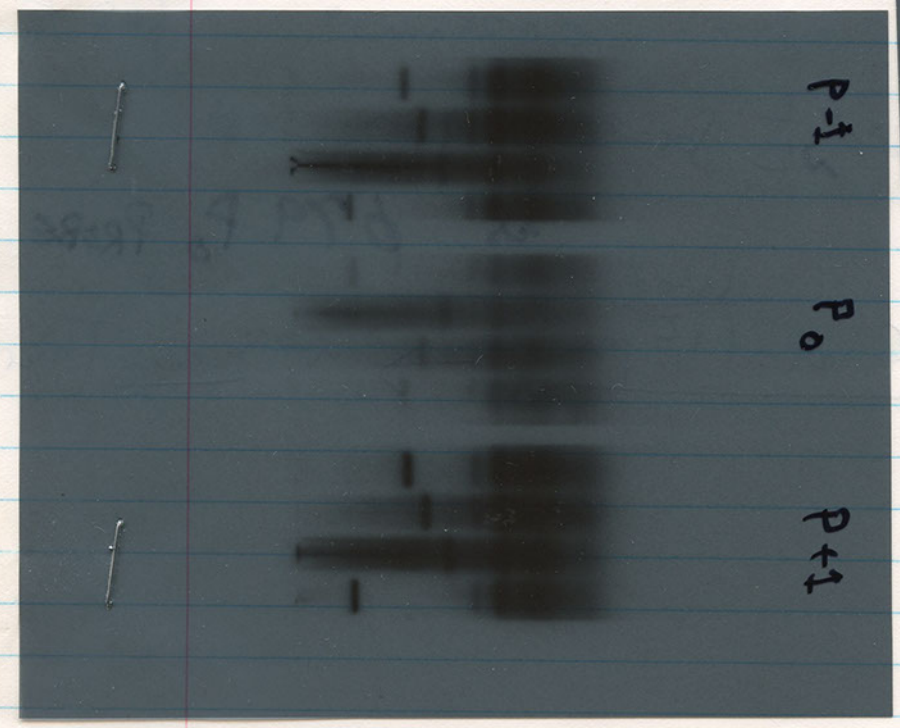
To 70°C at Wed 1.15 pm → ~9.30am Thurs



With cDNA probe at 70°C only one Hha piece can be found

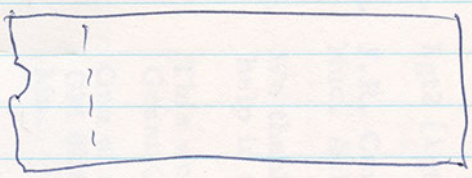
See also, 0130



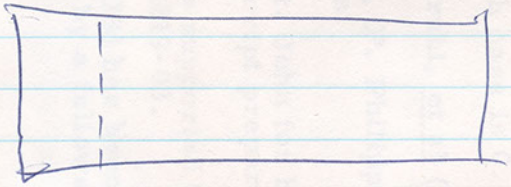


Thurs. Feb 24th

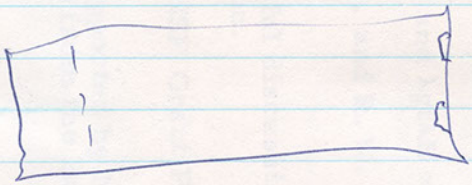
Probe test (contd.)



P_0 off. Used P_0 ϕ81 probe saved – it is yellowish.



P-1



P+1

Rinsed all in ~100 ml 2×SSC + 0.5% SDS to 70°C (together) at 10.45am

11.45am 11.55am → 12.25 1230 → 145pm

Rinsed in 2×SSC. Air dried + to autorad. at 2.20 pm Th. Off at 8.30am Fri 9.0am Fri → 9am Mon

Conclusion: Agarose method v. impressive but probe is doubtfully specific. ∴ repeat with S1 first. Try old DEAE-HCl method to clean up agar.

Screening λgt Recombinant Clones by in situ

Hybridization to Single Plaques

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ABSTRACT

A rapid, direct method for screening single plaques of λgt recombinant phage is described. The method allows at least 10^6 clones to be screened per day and simplifies physical containment of recombinants.

Recombinant DNA technology has made it possible, in principle, to isolate large quantities of DNA corresponding to any single gene. This isolation has been accomplished in any of three ways: (i) purification of the gene or its transcript before insertion into a vector for cloning (1); (ii) genetic selection of the desired gene after shotgun (2) cloning the genome of the donor organism (3); or (iii) using complementary RNA or DNA probes to screen clones produced through a shotgun experiment. The first method is limited by the practical difficulty of physically purifying the complete nucleic acid sequence of any gene. Application of the second is limited to genes for which appropriate mutations are available in hosts which can express foreign genetic information. The third method is limited only by the availability of complementary nucleic acid

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Screening methods have been developed for both recombinant plasmid (4) and bacteriophage (5,6) cloning systems. The most rapid of these, the methods of Grunstein and Kramer, are based on a technology developed by Olivera and Bonhoeffer (7) for growing and lysing cells on membrane filters. The speed and simplicity of these methods is limited by the need to pick and spot plaques or colonies from plates onto filters for further growth. We have found that a single plaque of a λgt recombinant phage contains enough phage DNA for detectable hybridization to complementary labelled nucleic acid and that this DNA can be fixed to a nitrocellulose filter by making direct contact between the plaque and the filter. These findings have allowed us to develop a method for screening

recombinant phage which has significant advantages over previous methods: (i) Physical containment of recombinant molecules (and organisms carrying them) is made simpler because this method requires fewer manipulations of viable phage, requires the growth and handling of much smaller quantities of recombinant DNA-containing phage and cells, and produces no contaminated materials other than the petri dishes on which the plaques are grown. (ii) At least 2×10^4 plaques can be screened per hour (8), which compares with about 100 per hour by the fastest previous method (6). (iii) Because up to 2×10^4 plaques can be screened on a single petri dish and nitrocellulose filter, the method requires smaller amounts of materials, including radioactively-labelled probe. (iv) Because screening can be done directly from the transfection plate, all the independently-constructed phage containing similar or identical inserts can be isolated and individually characterized. This can give information on the heterogeneity of repeated genes or of genes from different somatic tissues.

We believe that this method will be widely applicable and we, therefore, present it here in detail. Preliminary experiments and the model system used for optimization are also described.

Cells (9) are infected with phage, plated in L-soft agar on L-plates, and grown for at least 12 hours at 37°C. Large plaques are usually desirable and 0.1 ml of late log phase cells per plate has been found to give large plaques of many λgt phages. The number of phage which can be plated on a single petri dish depends on the relative growth rates of the desired recombinant and the background phages (see below). After growth, the plates are placed at 4°C for about 15 min to harden the agar. Phage and DNA are transferred to a nitrocellulose filter by placing the dry filter (88 mm, Millipore HA or Schleicher and Scheull, BA 85) on the lawn, being careful to form no air bubbles between the soft agar and the filter. Phage are allowed to adsorb to the filters for 1-20 min. If duplicate

filters are desired, several filters can be adsorbed sequentially for 30-60 sec each or filters can be stacked and adsorbed at once. If filters are stacked, they must be allowed to adsorb for at least 20 min. During adsorption, it is convenient to mark the filters and plates for orientation (see below). Following adsorption, the filter is carefully lifted from the plate to avoid removing the soft agar layer which occasionally adheres to the filter. Should this occur, the agar can be removed by gently shaking the filter during the denaturation and neutralization steps. DNA and phage are denatured and fixed *in situ* by dipping the filters in 0.1 *N*, NaOH, 1.5 *M* NaCl for 20 sec and the filters neutralized by dipping in 0.2 *M* tris, pH 7.5, 2 x SSCP (11) for 20 sec. Filters are blotted and baked at 80°C *in vacuo* for 1.5 to 2 hours.

Hybridization to the nitrocellulose replicas is carried out in a siliconized glass petri dish using 10^5 - 10^6 cpm of ^{32}P-labelled probe (10) per filter in enough 5 x SSCP, 50% formamide to cover all filters. Hybridizations are typically for 12-18 hours. Buffer and probe can be recovered and reused for at least two weeks. Following hybridization, filters are washed in a large (10-15 ml/Filter) volume of 5 x SSCP, 50% formamide at 42°C for 30 min. and then for 20-30 min in 2 x SSCP at room temperature. The filters are then covered with plastic wrapping film and placed against X-ray film for autoradiography. 24-48 hours of exposure is usually sufficient with a homogeneous probe of specific activity of 10^5-10^6 cpm/mg. X-ray intensifying screens (Kodak X-Omatic

been used and little difference noticed. Very long adsorbtions resulted in slightly more diffuse plaque-spots on

Regular) have been used with flash-activated film at -70°C to shorten exposure time.

The method described above should be generally applicable to all recombinants of λgt phages. The experiments described below used the phage λgt-Sc 1109 (6, 12) in model systems to optimize the method. Phage grown on E. coli ~~DX~~ C600 for less than 9 hours give negligible autoradiographic signals; 12-14 hours of growth is optimal. Although T-plates generally yield larger plaques, these apparently contain fewer phage particles and less DNA than comparably sized plaques grown on L-plates, since, at all growth times, nitrocellulose replicas of T-plates gave lower signal than those of L-plates (13).

To determine the maximum number of plaques per plate which can be screened under various relative growth conditions, two experiments were performed. In the first, to model a situation in which the background grows much better than the desired recombinant phage, a small number of λgt-Scl109 phage were grown together with increasing numbers of λgt2 (14) phage. No effect on signal was apparent up to about 2000 pfu/plate, but the signal was weak and variable with more than 4000 pfu/plate. In the second competition experiment, λgt-Scl109 was plated with increasing numbers of λgt-λB, which has approximately the same plaque size as the recombinant phage. In this case, sufficient signal was obtained with more than 2×10^4 pfu/plate. A practical rule seems to be that the method will accommodate the greatest number of plaques that does not produce confluent lysis.

Time of phage and DNA adsorption to the nitrocellulose replica filter does not seem to be critical; adsorbtion times of 1-60 min have

been used and little difference noticed. Very long adsorbtions resulted in slightly more diffuse plaque-spots on the nitrocellulose replicas and the autoradiograms. Stacks of at least five filters can be adsorbed at one time to produce replicates (for hybridization to several different probes, for example). For most applications, better replicates are produced by sequentially placing the filters on the plate for a brief adsorption to each (Fig. 1).

Dipping the nitrocellulose replica directly into a beaker of denaturing or neutralizing solution does not result in loss of signal or in distortion of the shapes or relative positions of the plaque spots. Denaturation and neutralization times of 0 to 60 sec all give acceptable results; 15-20 sec of each is optimal, giving an approximately 2-fold increase in signal over either no denaturation or no neutralization. Baking the filters before hybridization is not necessary, but in this model system the signal was increased approximately 2-fold by baking for 90-120 min.

Following autoradiography, it is necessary to align the autoradiogram of the nitrocellulose replica with the original plaque plate to allow picking of the plaques corresponding to positive signals. The most reliable system we have used for this alignment involves labeling the filters and plates during adsorption by puncturing the filter at 3 points around its circumference with a hypodermic needle and injecting 0.1-0.2 µl India

ink into the agar at each point. The ink diffuses very little during the 2 days normally required for hybridization and autoradiography and allows absolute orientation of the filters, autoradiograms, and plates. From a plate with more than 1000 plaques, we normally pick about 20 plaques from the area of each positive signal for rescreening and plaque purification. Once pure clones are obtained, the sizes and restriction spectra of the inserted DNAs can be rapidly determined by the method of Cameron, et al (15).

The large capacity and speed of this method make it practical, we believe, to isolate a eukaryotic unique gene by screening all the recombinants produced through shotgun cloning the entire genome. This should make possible isolation of a large number of genes for which physical or genetic purification schemes are not available.

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B.E. Noyes and G.R. Stark, Cell 5, 301 (1975);
S. G. Clarkson, H.O. Smith, W. Schaffner, Birnstiel, Nature

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   G. N. Yenikolopov, A.P. Ryskov, T. Nitta, and G.P. Georgiev, Nuc. Acids Res. 3, 2645 (1976);
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   R. Higuchi, G.V. Paddock, R. Wall, and W. Salser, Proc. Nat. Acad. Sci. USA 73, 3146 (1976).

2. A shotgun experiment is defined as one which involes the production of recombinant DNAs between the vector and total or partially purified DNA from a cellular source.

3. K. Struhl, J.R. Cameron, and R.W. Davis, Proc. Nat. Acad. Sci. USA 73, 1471 (1976);
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4. L. Kedes, A. Chang, D. Houseman, and S. Cohen, Nature 255, (1975); M. Grunstein and D. Hogness, Proc. Nat. Acad. Sci. USA 72, 3961 (1975).

5. K. Jones and K. Murray, J.M.B. 96, 455 (1975); Yu. V. Ilyin, N.A. Tchurikov, and G.P. Georgiev, Nuc. Acids Res. 3, 2115 (1976);
   B. Sanzey, O. Mercereau, T. Temynck, and P. Kourilsky, Proc. Nat. Sci. USA 73, 3394 (1976).

6. R.A. Kramer, J.R. Cameron, and R.W. Davis, Cell 8, 227 (1976).

7. B.M. Olivera and F. Bonhoeffer, Nature 250, 513 (1974).

8. This calculation is based on the worst case in which only 2000 plaques can be grown on each of 50 plates, the screening of which would require about 5 working hours over a three-day period.

9. E. coli C600 rk^- mk^+ was the usual host. Cells and phage are described in J. R. Cameron, S.M. Panaseuko, I.R. Lehman, and R.W. Davis, Proc. Nat. Acad. Sci. USA 72, 3416 (1975).

10. In the model experiments described, the probe was total yeast rRNA ([^{32}P]-labelled) prepared by the method of G.M. Rubin, Methods in Cell Biol. 12, 45 (1972).

ink into the agar at each point. The ink diffuses very little during the 2 days normally required for hybridization and autoradiography and the filters, autoradiograms, and plates.

11. SSCP is Standard Saline Citrate
1 x = 120 mM NaCl, 15 mM NaCitrate, 13 mM KH_2PO_4,
1 mM EDTA, titrated to pH 7.2 with NaOH.

12. λgt-Sc1109, the gift of R. Kramer, is a recombinant λgt phage which carries 2 EcoRI fragments of Saccharomyces cerevisiae DNA, one of which carries the 5·8 S and 26 S rRNA genes. Approximately 5% of this phage's DNA is complementary to rRNA.

13. T-plates = 1% Bacto tryptone, 0.5% NaCl, 1% Agar, made to pH 7.5 with NaOH.
L-plates = 1% Bacto tryptone, 0.5% Bacto yeast extract, 0.5% NaCl, 1.25% Agar, made to pH 7.5 with NaOH.
soft agar in each case is 0.7% in Agar.

14. λgt2 [λgti in Struhl, et al (3)] and λgtλB were the gifts of M. Thomas.

15. J.R. Cameron, P. Philippsen, and R.W. Davis, submitted to Nuc. Acids Res.

16. We thank T. St. John for helpful discussions and Lynn Horn for help in manuscript preparation.

17. This work was supported by NSF Grant PCM76-02600 and NIH Grant GM·021819-03.

One of us (WDB) has been supported by NIH Training Grant 5T01-GM 01156 and by a fellowship from the Bush Foundation, St. Paul, Mn.

Fri Feb 26th

Clean up of agarose

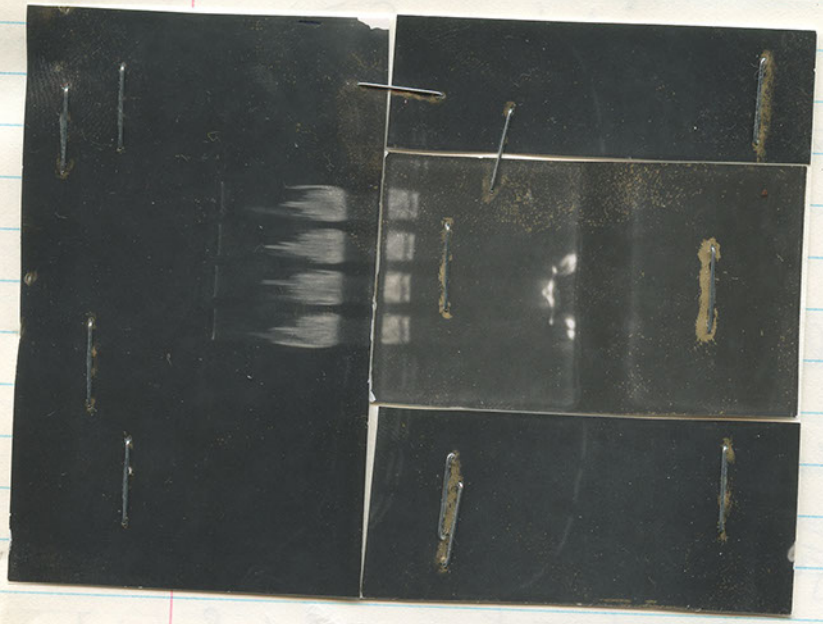
DEAE 23 A brand of Fibrous ex. Whatman Acidified with ~1M HCl ~2M NaCl → washed with d.w.

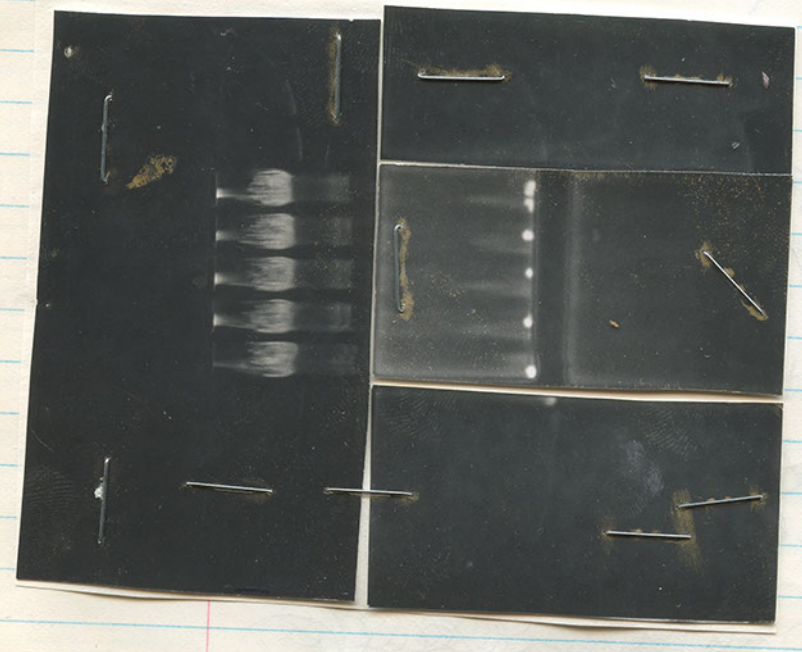
Recycled with 0.5M NaOH → d.w → repeated once more. Finally acidified to pH 1 with HCl alone, d.w. washed to suspended pH ~4 → fried at 42° until Monday am.

0.7% agarose made as usual with 4X Hemmings except Millipore finally. (For boxes) 0.75g agarose + ~50ml d.w. (+ 1.5g moist DEAE.HCl after first boiled. Millipore 0.22. Added 20X buffer → d.w. to usual.

R1 digest of 3A available from → B (has $\frac{14}{23}$ µg/µl ie 0.6µg/µl) — not enough!

Clare [illegible] + 40µl HX buffer



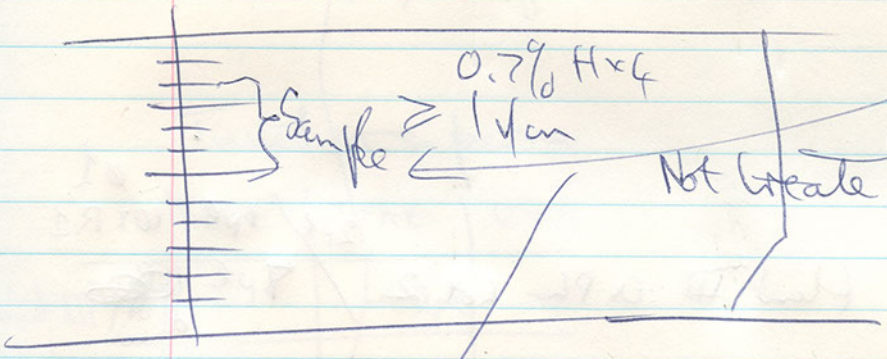


New digest: —

{ 20 µl ADA 19 Ch3 (+lac) DNA (≅ 14 µg DNA)
+ 2.3 µl 10× R1 + ~0.4 µl DGR1
to 37° at 1.40 pm → 2.30 pm

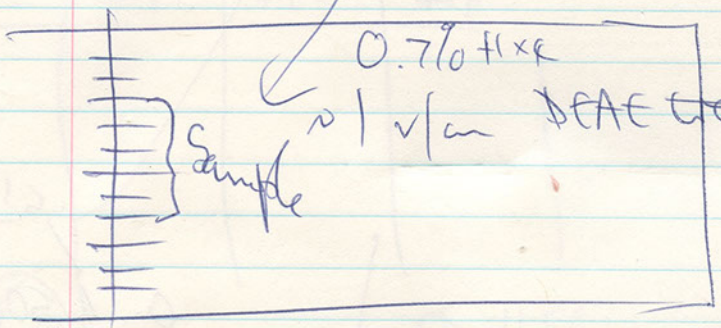
Diluted with 50 µl d.w.

0 ~ 2.45 pm



~ 1 v/cm

Off 11.45 am



~~First cut off origin~~ 2 cm. from each stained. Then edges. Then correct band. Probably OK – but tended to over-estimate migration.

1 pm – two pieces washed in 5 ml 1× R1 buffer with gentle rock for 15 mins. to get to R1 salts (? in real test run gel in nick translation salts)

~ 1.15 pm

Lot 2

2µl / 1µl ø1 10xR1

1µl d.w

5.10

↑ ka 6½V

↓ ka >0

On 4.50pm

3V/cm

H×4

0.7% Agarose

D+E

D+E

U+E

U−E

Decanted liquid - Freeze

Mon Feb 28th

Cont^n of DEAE test

Thawed gels & collected ~~20~~ ~200 µl of super^t from ~~a lot less~~ "usual" (~10µl was lost)

~300µl from "DEAE"

Refreeze gels

Refreeze super^ts

Tested ~~on~~ supernatants :—

	DEAE	DEAE	USUAL	USUAL
Hind III	—	+ 2µl	—	+ 2µl
Super^t Sample	40µl	40µl	40µl	40µl

11.10 am to 37°

↓

11.40 am

~20µl of each iced — rest rm

On 11.50 am

Off 1.00 pm

0.7% Agarose

H×4

3V/cm ~100mA

Rerun 1.40 pm

↓

2.40 pm

1.30 pm back to 37°

↓

4.30 pm

Modification in day in lab

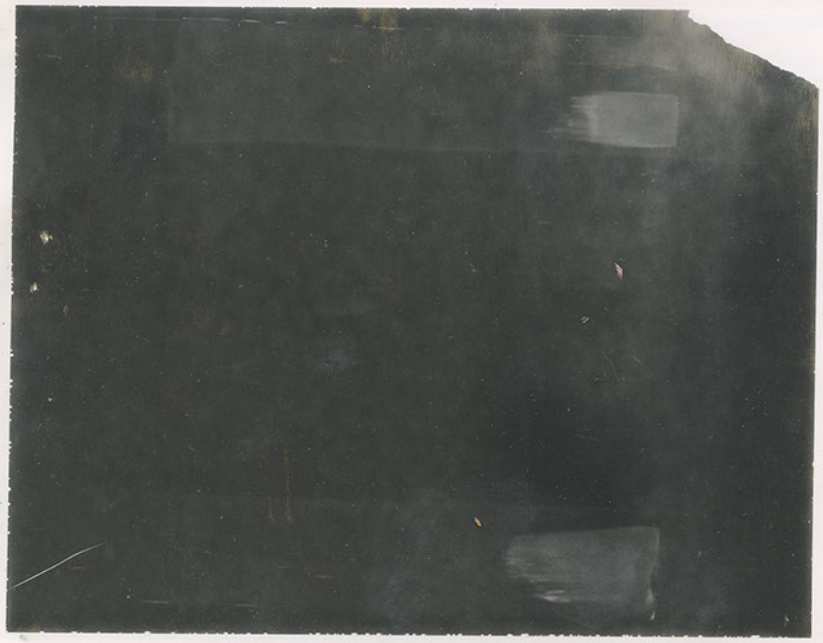
DEAE − enz

DEAE + enz

USUAL + enz

USUAL − enz

Odd result !



Mon. Feb 28th

Nick translation from gel

Can titrate any gel troubles with more enzyme.

Decided to try :—

Run R1 of Ch3A to get lac.
Freeze gel after dialysing to nick trans^n buffer (unless I can run in it).
Add dNTP's (α-^{32}P), DNA pol. Incubate.
Add cold pyrophosphate 6xSSC etc.
Denature + hybridize.

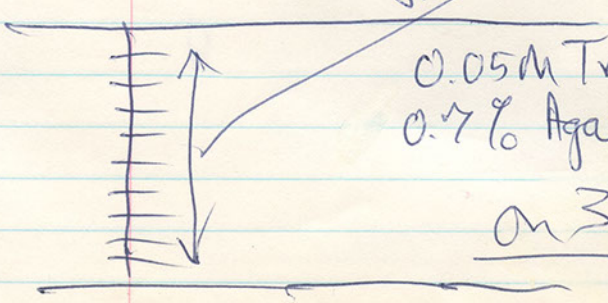
12.20pm { 10µl ADA 19 Ch 3(+lac) DNA (≅ 7µg DNA + 0.7µg lac)
+ 1µl 10xR1 (φ1) + ≈0.2µl DGR1

Off 1.10pm → to ice. Diluted with 110µl 1xTris/EDTA

Nick translation buffer is

0.05M Tris pH 7.8 } Run in this with 0.001M EDTA

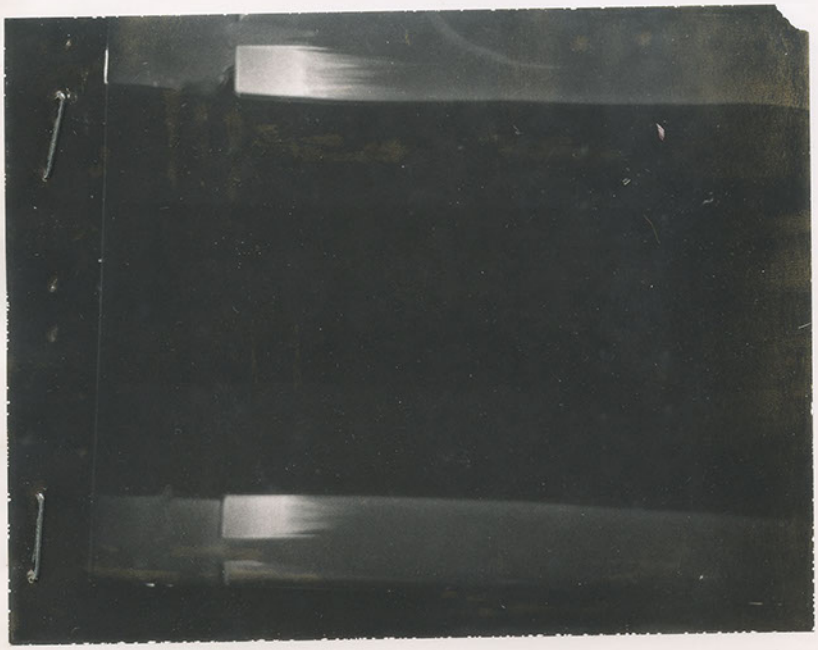
Make 7 to take care of EDTA → 5mM $MgCl_2$
1mM ME
50µg/ml BSA } Add these as 10x later



0.05M Tris HCl / 0.001M EDTA pH 7.8
0.7% Agarose / DEAE brake

On 3pm 1v/cm Off 8.15am

Offer still



Tues. March 1st

Nick translation again

Can't understand .05M Tris HCl / .001M EDTA being so poor an e.p. but can go back to Hennings 1X :-

.04M Tris. acetate
.02M Na. acetate
.002M Na_2 EDTA
.018M NaCl
} pH 8.05

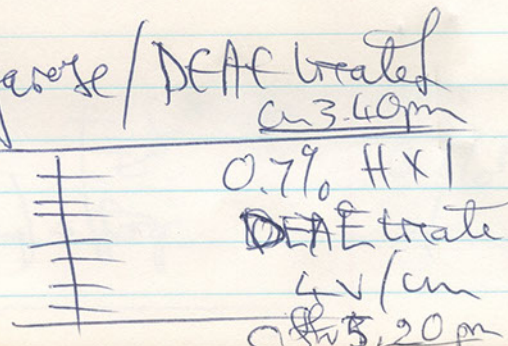
& adjust it to pH 7.8. Then diluted to ½X with equal vol. of d.w. containing 2mM ME
100 µg/µl BSA
9 mM $MgCl_2$

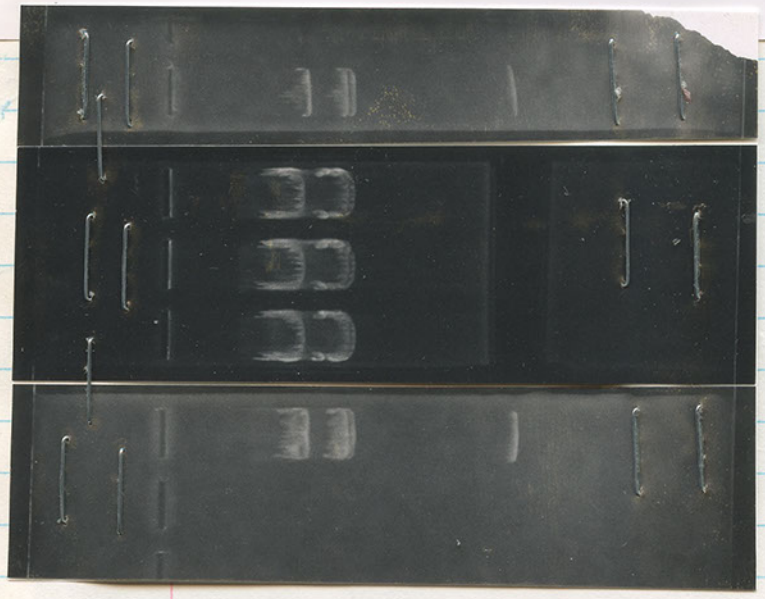
it sh'd approximate nick translation buffer

∴ repeated digest 2.40pm to 37° } 10µl ADA 19 Clarion 3A
1µl 10× R1 (ΦK)
~0.2µl DG R1

3.15pm (R.T.)

Remate 0.7% Agarose / DEAE treated in 1X H





Wed - March 2nd

Repeats of recent gels

ADA 19 Ch 3A	10 µl	10.30am to 37°
10x R1 buffer	12 µl	
DG R1	~0.4 µl	
D.W.	100 µl	

1.30pm ~4v/cm in following systems:-

0.7% Agarose

- - - - - DEAE treated Tris .05M / .001M EDTA
- - - - - - - - - - - - - - - - - - H×1
- - - - - Not treated H×1
- - - - - - - - - - - - - - - - - - H×4

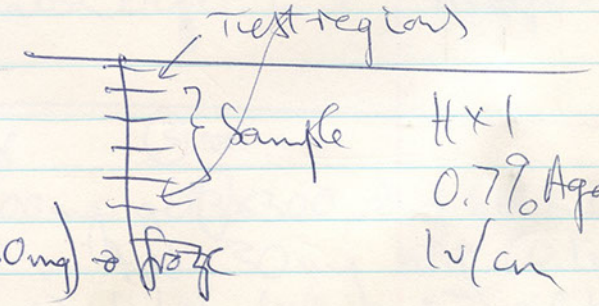
Off to R. 0pm

Confirms DEAE is trouble if buffer is not high.

∴ Test Tris. HCl / EDTA straight.

— but meanwhile test available H×1

On 5.25pm



Off 9.0am

Cut out band for lac (330mg) → frozen

Thurs March 3rd

Further nick translation ideas

Since ep. is poor at low salt it does not look good to try the original idea

— i.e. gel ep, freeze, nick translate, hybridize

This is ok only if probe is already hot i.e.

gel ep., hybridize. ∴ try Kelly's procedure

(see ϕ74) which is to dissolve gel in 6M KI

10mM Phos. pH7 & a least to small Hydroxyl

Apatite column. Wash with KI to remove agarose

& with low phos. to get rid of KI.

Then elute with high phos. Still would need

to dialyse or Sephadex

Before giving up my own idea, try adding solid

KI to (frozen) gel & running over small Sephadex

column with translation buffer in it. As expected

— it clogs.

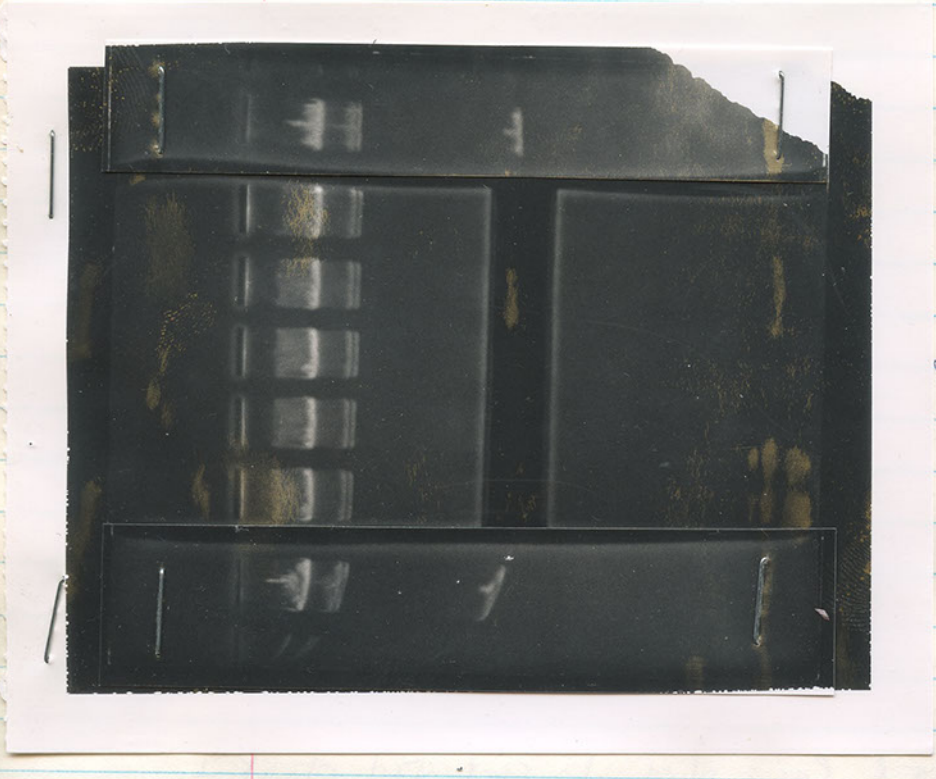
∴ try :— (1) vol (3) vol

Add to e.g. 300mg gel (3)x300 µl of 6M KI

10mM Phos. pH7. Add 30mg HAP (0.1 vol)

(Capacity is ≈1mg DNA / 1ml swollen gel) Wash (1x by cent.) with KI

∴ ≅ 30µg



So it works.
Need 32 P for yield

~ 60 µl

Wash 2x with 10mM phos. Take up in 0.6M phos. to give
0.3M → apply to top of small Sephadex against
1mM EDTA buffer. Collect using precalibrated volumes. Ppt.
with Ethanol if reqd.

---

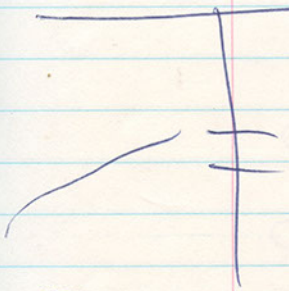
095 hac gel (330mg) + 1ml ~6M KI (sat.d) K Phos
~10mM. + tad DTT to keep I_2 down

+33mg dry HTP (Bio Rad)

Shid at Hut 1st

Vortexed well. left ~10min
— — again. Cent.d Decanted.
Washed 1x with KI/phos. Cent.d Decanted.
Washed with 10mM K Phos 2X
Eluted with 650µl 1M K_2H / KH_2 } Phos.
(Final vol. ~150µl)

On 4.15pm → 5.15pm



0.7% Agarose
4xH
3V/cm 100mA (max)

Repeats —

On 5.20pm
0.7% H x4
1V/cm
~~#~~ 9.20am

ADA 19 Ch3A 10µl
10xRI 12µl
DGRI ~0.4µl
D.W. 100µl

4.25pm to 37°
↓
5.10pm

Friday March 4th

1.01g sample from lac gel 099
frozen & thawed

+2 ml Fresh ~6M KI 10mM KH_2/K_2H Phos. / 0.1mM DTT
(sat? is slightly less)

≈6500 cpm

Added to it (for tracer purposes) 2 µl of
LAF nick-translated Hha
Ch 3A Δlac which is now ≥ 1.5×10^6 cpm/µg
– so only ~ .004 µg total

12 noon ~ 30mg HTP obtained from 30mg washed 2x
with 1g settle with KI / phos.

left rocking until 2pm
Washed 2x KI
2x 10mM phos to vol. 150µl
Added 150µl 1M KH_2/K_2H Phos
Sephadexed to 0.001M EDTA

Salt came in tubes

∴ pooled 2 thru 7 (can't find ^{32}P)
Missed! Salt ppt'd ∴ repeat
with more ^{32}P

108 ≡ 324
72 ≡ 216
36 ≡ 108

Sat. March 5th

Recursiferation

It is clear that the S1 method on AT is not v. good.

It is also clear that ~~double label~~ nick trans.n on an S1 piece is not going to be easy.

∴ alternate generation.

Take our putative β chain clones.

Cut with Hae III :—

26	27	98	99	134	135
Glu	Ala	Val	Asp	Val	Ala

216bps ↑ 108bps

Hae III — In all β chains

Bam (Prob.y not in a V N) but expected in mouse & in our clone

Hae III — In all β chains

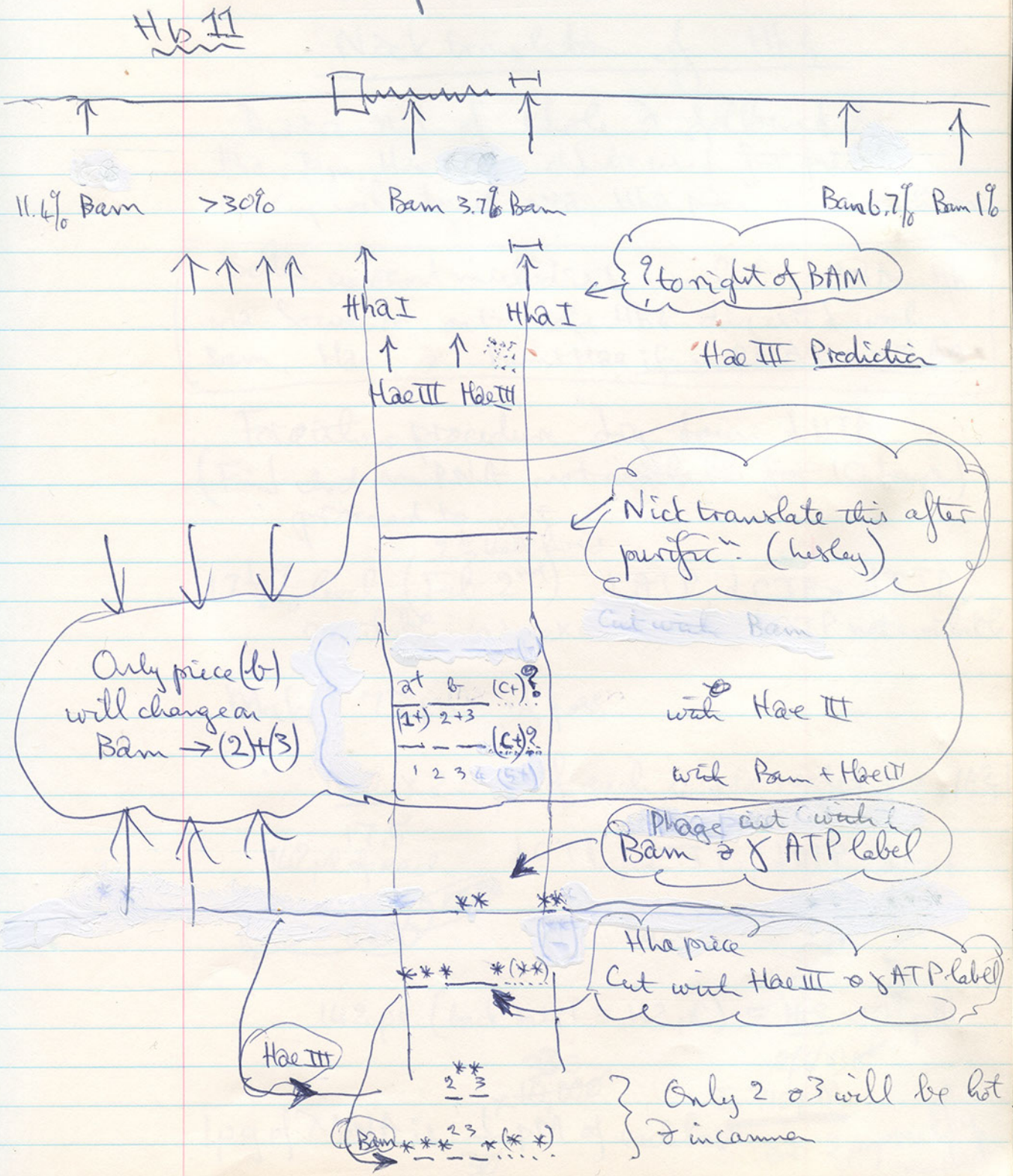
R1 site at 120 121 122: Lys Glu Phe. Probably not in mouse. Lys Asp Phe on in alpha

Then reclone in EK1 plasmid with no homology to lambda using R1 site cleaned up with S1 & but ligased. Detect reclone with cDNA from mouse Hb

Reclone in plasmid & detect
with cDNA from globin

If either HaeIII site is lost it
will still work on piece 2 or 3.

Present maps are :—



Wed. March 9th

Nick translation of Hb4

Direct n.t. of whole λ, followed by Hha, Bam, Hae etc. c'd be used for piece. Lesley will do it via Hha piece nick translation.

Better current method is to use hot cDNA Hb via Southern onto cold Hb4 digested with Bam, Hae, & Bam + Hae if mRNA is available

For either procedure try fresh dNTP's (Find that mRNA not available for 10 days) ∴ proceed to n.t.

$17\frac{1}{2}$% level (Feb 2nd) $2\frac{1}{2}$ half lives dATP, dCTP & dTTP available (as excess) dGTP not available

Make 75 μC if poss.

∴ $\frac{100}{17.5} \times 25$ μl of each will be needed i.e. 142 μl

142 μl of each dCTP, dTTP, dATP dried

~~i.e. 200~~

Each is 200 C/mM of

∴ 142 μl (back dated ≡ 142 μC) ≡ $\frac{142}{200 \times 10^6} \times 10^{12}$ pM ≡ 710 pM

1 μg of λDNA is $\frac{1}{30} \times 10{,}000$ pM of each base or 2 μg really needed.

∴ take up frief form dNTPs in

90 µl HF 9-133 1X nt. buffer
(100 µl stock 10x + 800 µl dw + 0.7 µl MG)
+ 10 µl 3AHb4 DNA (1.5 µg)
+ 0.1 µl 20 mM dGTP (2000 pMoles)
+ 2 µl DNA pol. m151

→ To 15°C 12.20 pm

1.20 pm +10 µl 1% DEPC

Sephadexed to 0.001M EDTA pH7.8

	mR/hr		mR/hr		mR/hr
1	<0.1	4	13	7	1.7
2	<0.1	5	17	8	1.1
3	<0.1	6	4	9	1.1

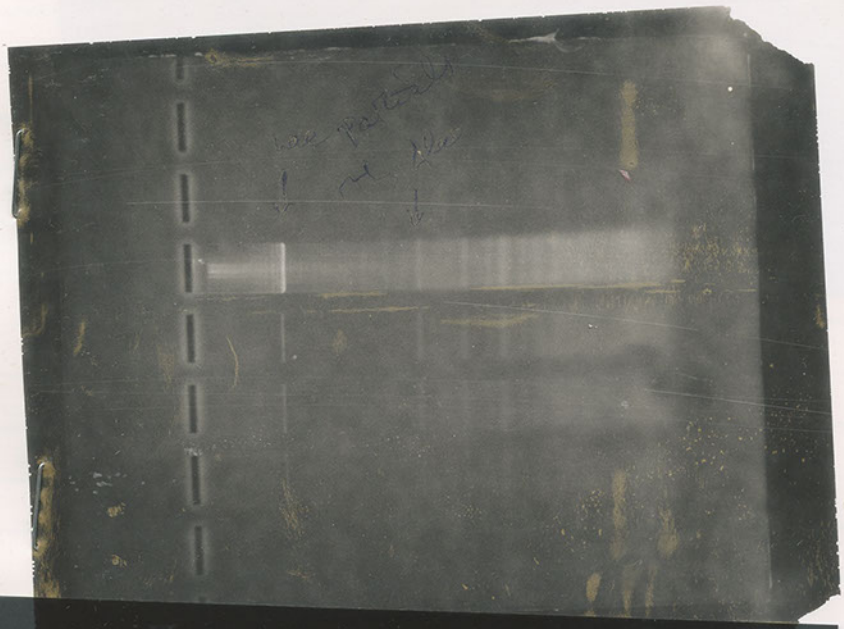
0.09
32P ChHb4

Pooled Vol. ~590 µl 5 µl ≡ 32,000 cpm

∴ incorp. ~1.6 µC

1 µl ≡ 6,000 cpm

Hesley has Ch3A at 18,000 cpm/µl



Hg
Arc
Hot

Thurs. March 10th

Hha digests

			20µl	20µl	20µl	Rest
10 x Hhabuffer	✓	8 µl				
ADA 19 (3A)	✓	2.5 µl				
10-113W (Δlac)	✓	3 µl				
0109 32P Hb4		—		6µl ✓	12µl ✓	
hesly 32P (Δlac)		—	2µl ✓			
D.d.w.	✓	53 µl				
Hha enz. Batch 139 Biolabs	✓	4µl				

To 37° 1.30pm → 2.40pm

On 2.50pm Off 9.30pm

D.W.		Stained
Cold mix (resistive)	1.5% Agarose	Fridge + exposed
Hb4	HX4	10pm → 12mon
3A Δlac	1.5 V/cm 60mA	Th 24hr Fri
2 x Hb4		Fri — Sun
Resistive 32P labels		Noon 48hr Mon
DW.		

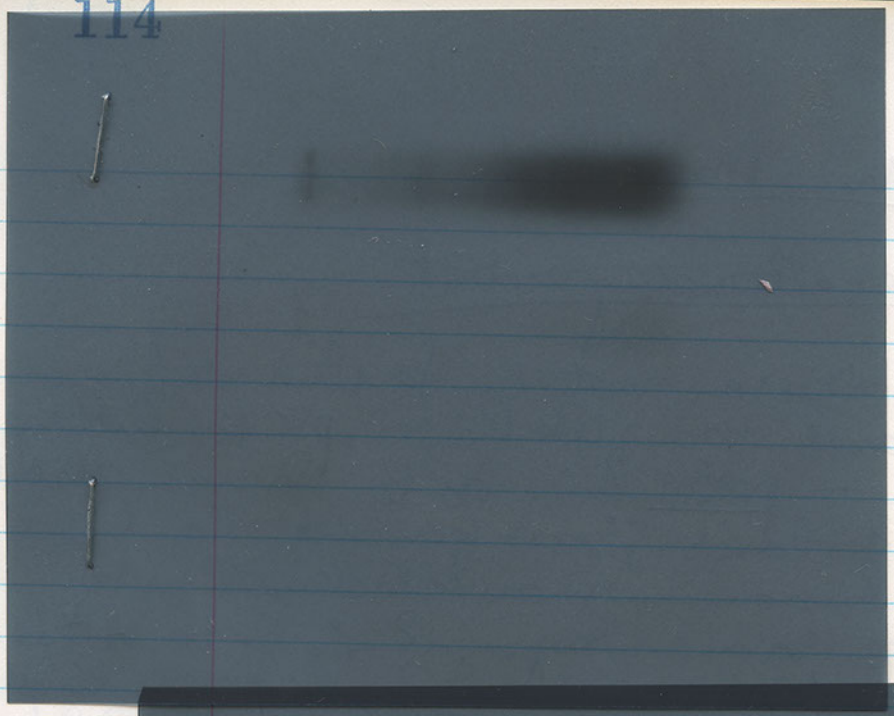
Product is adequate!

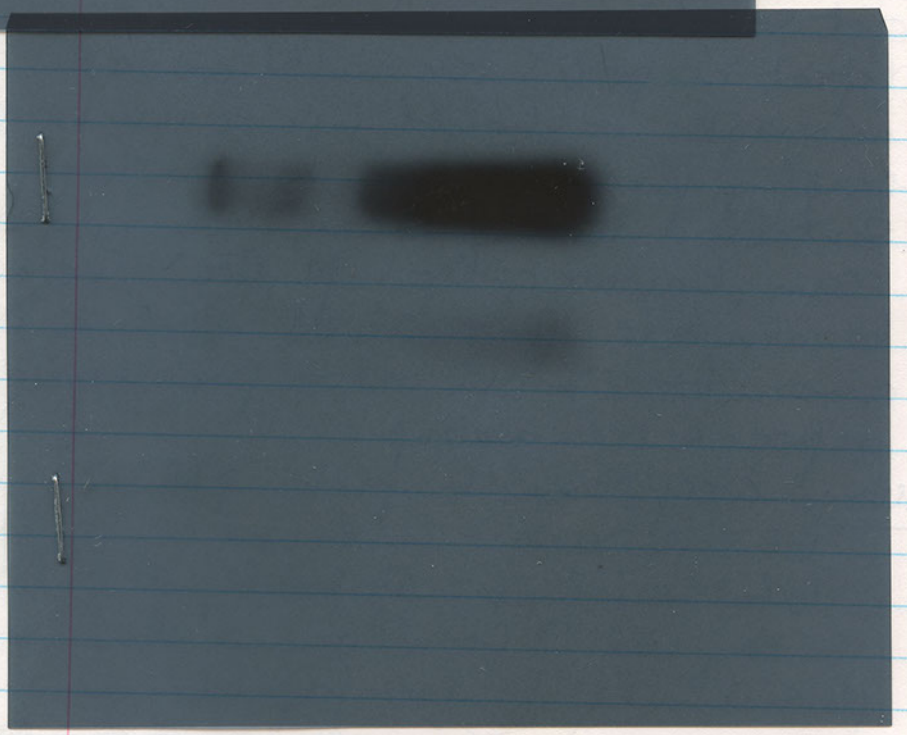
Mon. March 14th

Re-alternating vectors

No reason not to do this with histones too. We have pSC101 histone clones — probe? ∴ go to λ immediately for R1 clones from genome. Can test the human & mouse R1 clones also in the same way. ? ∴ go to 4A to get selection v. screening — except length of repeat is prob'ly too small for 4A. ??
∴ may be we should use a non-cross reacting plasmid with no homology. With suitable DNA conc.ns the self-ligating can however be kept low. We need to calculate efficiencies & total DNA amounts & in vitro packaging results & pre-screen grow up

After discussion: Every reason to pick pBR322, — it is EK2 in N776 & can be used semi-selectively to but-end ligase. We probably will find it best to stay with λ for all genomic clones, & use plasmid only for probe preparation once cut-out-able pieces are in use. It is also probable that sphero + DNA → 1 round lysis (100 copies) will be best, since we can plate up to 20,000 plaqs but only 1000 sphero plaques/plate, also v. ~2000 bacterial colonies/plate.





Cut out putative band from Hb4. Frozen.
Rest re-autoradiographed.

Thurs. March 17th

Remake of Hha piece (^{32}P) for HaeIII

Make up 1.5% agarose gel with H x 1 buffer so as to be able to get HaeIII to work after extraction.

Digestion shld be an ppt. wick translated to get plenty of counts in small vol.

~300,000 cpm — take 50 µl Hb4 0109 & add 150 µl EtOH — went a little cloudy

~900,000 cpm — — — 50 µl Δlac D101 B (Hershey) — — — both in 10^{-3} M EDTA

✓ 2.30 pm to −70°

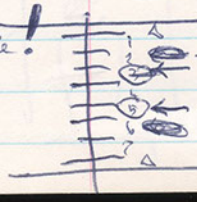
10 x Roberts 60 mM $MgCl_2$
60 mM Tris pH 7.9
60 mM NaCl

✓ 2.45 pm spun in Beckman B (10,000 rpm) for 5 min
✓ Blow off EtOH
✓ Add 2.5 µl 40 mM $MgCl_2$ to each (to neutralize EDTA)
✓ 20 µl to each of { 5 µl 10 x Hha i.e. Roberts buffer; 2 µl Hha enz. Batch 139 Bos labs; 4 µl DDW }

By Geiger there are ≥2 x more counts in Hb4 sample!

3.35 pm to 37°C Off ~4.35 pm

Off 5.30 to autoclave 10.30 pm



On 4.40 pm
1 x H
1.5% Agarose
1.5 V/cm
12.15 am

Tubes

1	<0.1	5	0.7 mC/hr
2	<0.1	6	0.7 mC/hr
3	<0.1	7	0.1
4	<0.1	8	0.1

Tomorrow label

Just possible that counter was wrong — it quit shortly after

I must have muffed one back!

— no — that is not it.

~~A shit day for mistakes!~~

Fri. March 18th

Remake of ^{32}P plasmid

Result not v. good on Hha 2 piece ∴ remake nick translated products again — altho' it's done directly from Hae III & select by cDNA (globin).

Take 2x80 pC total (20 pCi of each dNTP) per reaction

— today this is 87 pl of each — by error & took 174 pl ∴ save $\frac{1}{2}$ as {Equimolar Mix dNTP's March 2nd lot} ~ {400 pl

~~Same error here~~

~~$\frac{1}{2}$ was < 400 pl~~

~~100 pl~~

O.K.

Take up in ~~200~~ pl of

200 pl
4 pl DNA pol m151

{100 pl {Stock 10x
800 pl d.w.
0.7 pl ME

HF9-133

~~100 pl~~

To ~~rest~~ whole, added

~~7 pl~~ 3A Hb 4 (~1 pg)

~~pl~~ 2 10-113 Y (~1 pg) ← Harvey's CR3A Δlac DNA

[∴ just do Δlac]

Can't find it — dammit!!

To 15° at 12.45 pm.

DEPC (10 pl of 1%) at 1.45 pm

G100 to 10^{-3} M EDTA pH 8

— no — this is not it.

Fri. March 18th.

Test of Hha Hb 4 piece

0119 Digest Δlac 3A

Marker Standard Hha:—

10 x Hha buffer	6 µl ✓
10-113 W (Δ lac)	4 µl ✓
ADA 19 (3A)	2.5 µl ✓
D.d.w.	44 µl ✓
Hha enz Batch 139 Boehlabs	4 µl ✓

To 37° at ~~1.20pm~~ 2.20pm

+ DEPC to 0.1%

Thawed out 0115 piece — 1 µl sieber gave 126 cpm − 15 cpm background ~120 cpm

Added 100 µl d.w. − to ice.

3pm Refreeze ~~think~~!

left overnight

5 µl gave ~ 55 cpm over background

No good.

1	<0.1	6	12
2	<0.1	7	33
3	<0.1	8	9
4	<0.1	9	~0.5
5	0.16	10	~0.5

Pooled 1,115 µl

5µl 40,300 cpm

4µC

8000 cpm/µl

Sat. March 19th

Repeat nicktranslation of Δlac
March 2nd lot

60pl of 4 base mix from o117 dried down (i.e. 25pC – shld be quite safe)

Took up in 90pl (w/pg) → { 100pl HEq 1-33, stock 10x nt buffer, 800pl d.w., 0.7pl ME

+ 2pl 10-113Y

+ 2pl DNA pol m151

To 15°C at 1.05pm DEPC to 0.1% at 2.05pm

G100 to .001M EDTA

So I poisoned the reaction with isotope yesterday

o121 Δlac 8000cpm/pl

Sat. March 19th

Another scheme

Take Hb 4, digest with HaeIII & with HaeIII + Bam, run on 1.5% agarose, transfer to Millipore, hybridize to cDNA of find desired piece. Problem – resolution of 300 b.p. pieces will be rather poor. Use marker & try it anyway.

Tues. March 22nd

HaeIII digests from hAF :—

(2) Hb4 – P3 G31 8 μg in ~50 μl with Roberts buffer (not inactivated)

(4) Ch3A Δ lac G422 7.5 μg in ~50 μl with Roberts buffer (not inactivated)

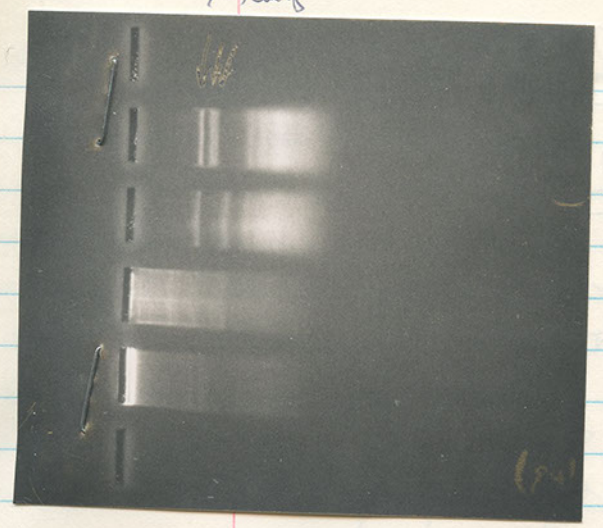
∴ digested :—

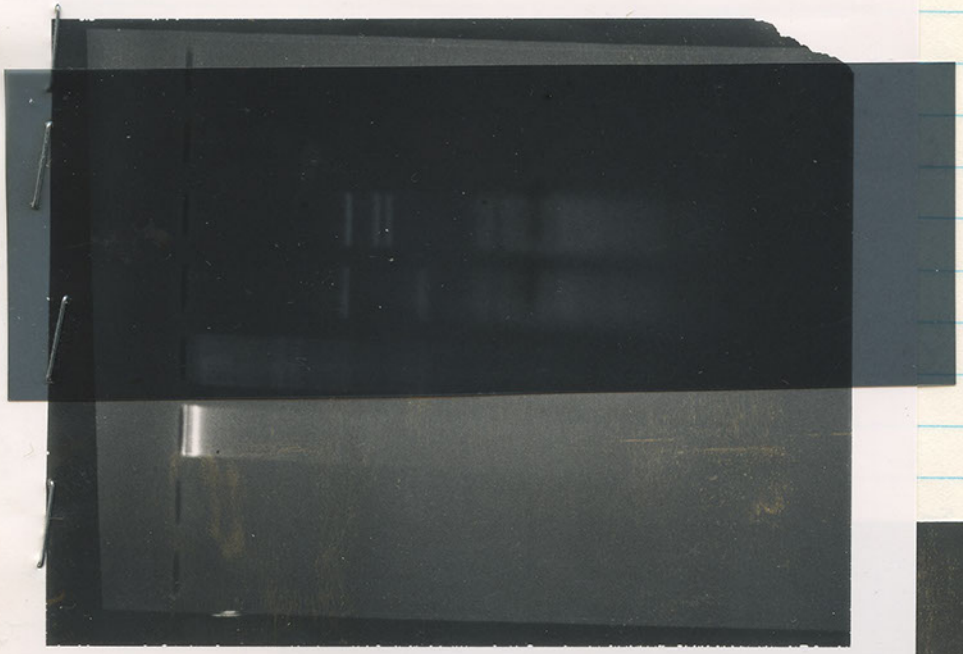
	Hb4 Hae	Δlac Hae	Δlac Hae/Bam	Hb4 Hae/Bam
(m93) 10X Bam buffer	8 μl	8 μl	~2 μl	~2 μl
(m7) BAM H-1	–	–		
d.w.	48 μl	48 μl		
→ (2)	24 μl	–		
→ (4)	–	24 μl		
			40 μl	40 μl

Save ½ for tasting

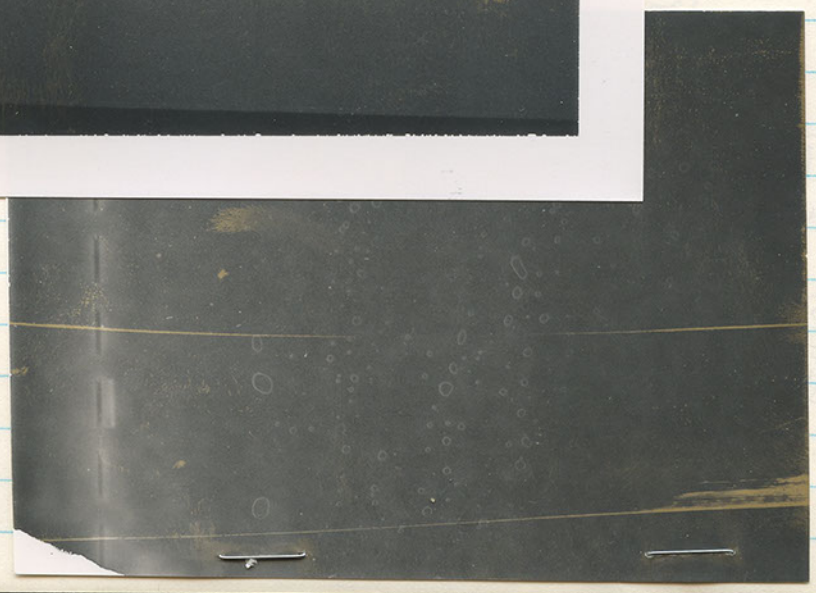
to ice 4:30pm — To 37° 4.30pm — 5:30pm ice

3 probable pieces
cut by BAM — common to Δ lac.





Should be only
5 BAM cuts.
I can see 3 of
them



Conclusion looks as if Hae III cuts done anyway & Bam makes a small piece too small to stick, & so fund.

6.0pm

On [redacted] Off 10.30 pm

1.5% Agarose
H×4
0.75 v/cm
Lagged
33 mA

Hb4 Hae III
Hb4 Hae III / Bam H1
Δlac Hae III / Bam H1
Δlac Hae III

} 0123

Stained Photographed. Resoaked in H×4.
Back on at 0.75 v/cm at 11.10pm 25mA
(so not completely back to H×4, but okay).

Off 9am

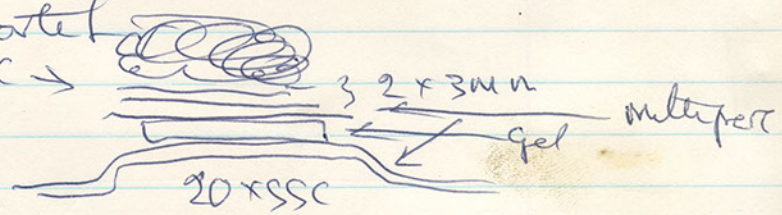
Hae III Δlac digest is poor

~9.15, 9.30, ~9.45

Sol^n I { 3M NaCl, 0.5M NaOH }

Sol^n II { 3M NaCl, 0.5M Tris pH 7 }

10.10am Transfer started



Flow seems rather fast; I am suspicious of a by-pass.

2pm Took off towels & replaced with smaller. Can't see by-pass — but flow became reasonable 10.19pm

7.30pm Rinsed 2×SSC ~20min. Dried. { Oven for 2 hr. 80°C } 8.10pm

10.25pm To 70° in Denhardt's Sol^n (cont^d on 131)

1	<.1	6	<.1	
2	<.1	7	7mR/hr	Pooled
3	<.1	8	33 mR/hr	1050 μl
4	<.1	9	8 mR/hr	
5	<.1	10	0.7	5μl ≡ 36,700
				7300

0127 7300cpm/μl
32P Hb4
nt

Wed. March 23rd

Nick translation of Ch3A Hb4

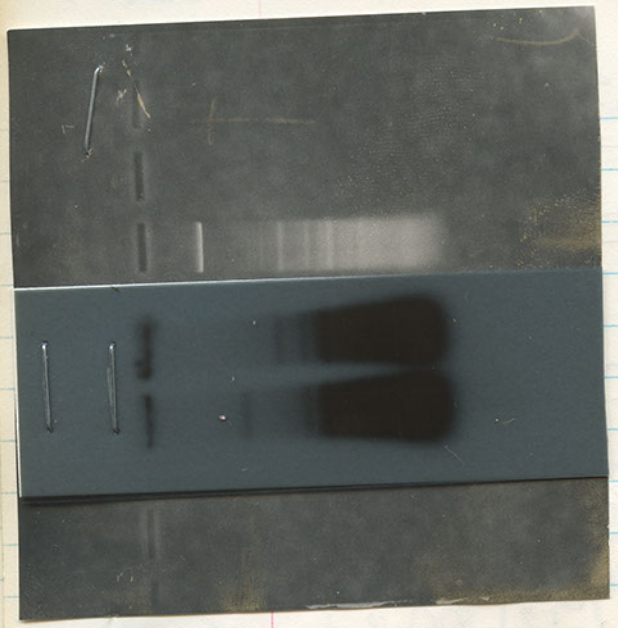
72 μl of 4 base mix from o/117 freezer down (i.e. ~25μC)

Took up in 90μl
+ 7μl Hb4 DNA (1μg) O.D 3.5 ex LAF G31 3/77
+ 2μl DNA pol M151

100μl HF 91-33 Stock 10x nt buffer
800μl d.w.
0.7μl ME

To 15°C at 11.10 am 0.1% DEPC at 12.10 pm

To .001M EDTA by G100 Sephadex.



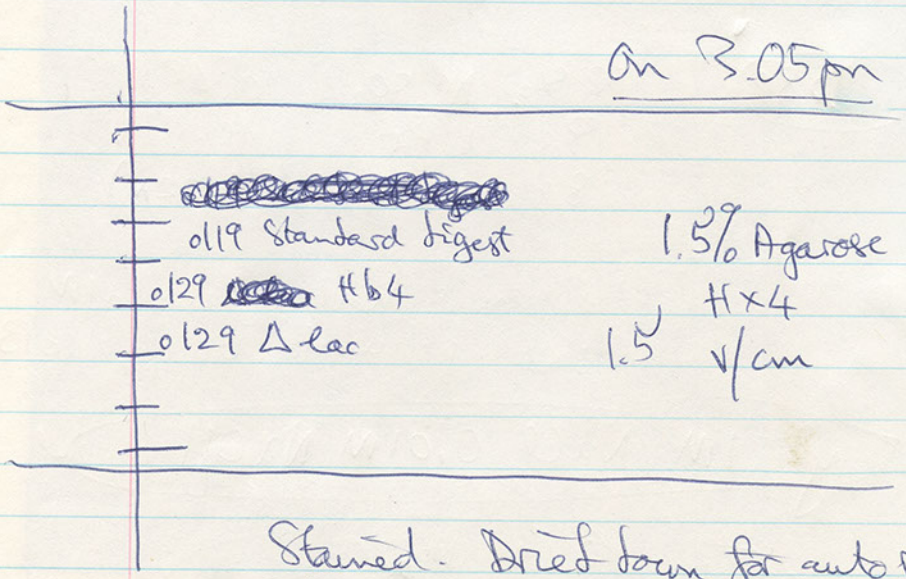
Wed. March 23rd

Hha of labeled Δlac → Hb4

	Δlac	Hb4
10× Hha buffer (i.e. 10× Roberts)	20 μl	20 μl
0127 Δlac 8000 cpm/μl	180 μl (1.4×10⁶ cpm)	—
0127 Hb4 cpm/μl	—	180 μl 1.3×10⁶
(See 072) Hha exp Batch 139 Biolabs	2 μl	2 μl

To 37° at 1.45 pm; 0.1% DEPC at 2.50 pm

On 3.05 pm off 7.30 pm



Stained. Dried down for autorad.
On 8.55 pm → 11.15 pm
11.20 pm → 8.45 am

An important background decreasing trick may come out of this accident — in which multiplex went into developer. As far as I recalled, it was washed in 2 SSC (probably not d.w.) after this. background is ~zero when developer was there. Try developer itself first. Then try pH 11.9 (pH of developer) + salt conc^n. of 39 mmho (≅ conductivity of developer). Effect is prob. high pH close to but less than that of dissociation of DNA

Typical KOH developer for high contrast

3M) ⇐ pK_1 ≃ 10

1 M)

20M)

to 14

(0.42M)

(0.07M)

(0.22M)

is also ≃1M

be close to 13

Try 1M NaCl 0.01M NaOH — this is pH ≃ 11.8 & cond.^{y} 41 mmho with 50 ml

or Try 0.4M Na_2SO_3

0.2M K_2CO_3

NaOH to pH 11.9

cond.^{y} is 39

An important background decreasing trick came out of this accident – in which multiplex went into developer. As far as I recalled, it was washed in 2×SSC (probably not d.w.) after this. Background is ~zero where developer was there. Try developer itself first. Then try pH 11.9 (pH of developer) + salt conc^n. of 39 mmho (≃ conductivity of developer). Effect is prob^y high pH close to but less than that of dissociation of DNA

Typical KOH developer for high contrast

	25g	Hydroquinone	(0.23M) ← pK_1 is 10
per litre	25g	K_2metabisulfite	(0.11 M)
D153	25g	KBr	(0.20M)
	50g	KOH	(0.89 M)

∴ total ionic strength is ~1M but this would have a pH close to 14

Perhaps better formula is

D82

	52.5g	Na_2SO_3	(0.42M)
	8.8	KBr	(0.07M)
	8.8	NaOH	(0.22M)

Total ionic strength is also ~1M but pH would be close to 13

Try 1M NaCl 0.01M NaOH – this is pH 11.8 & cond^y. 41 mmho with 50ml.

or Try 0.4M Na_2SO_3 0.2M K_2CO_3 NaOH to pH 11.9 → cond^y is 39

Thurs. March 24th

cDNA Hb probe last of gels

0.25 Millipore → 1/5 0.73 Millipore

in sack + 1000 µl (~1×10⁶ cpm.) of Harvey's
HF 11-49 cDNA probe + 2 ml Denhardt's Hybridizing
~~Annealing~~ Sol. + 1 ml 0.57 2X Hybridizing

To 70°C at 1.30 pm

Save this probe. Recovered 3.5 ml

11.45 am to 70°C with 2×SSC ½% SDS.
↓ Fri, after rinse
12.45 pm → 1.40 pm → 2.40 pm

Rinsed 2×SSC. Dried. To autorad 3 pm
Off 1L Can

Unfortunately Millipore stuck to film (not quite dry)

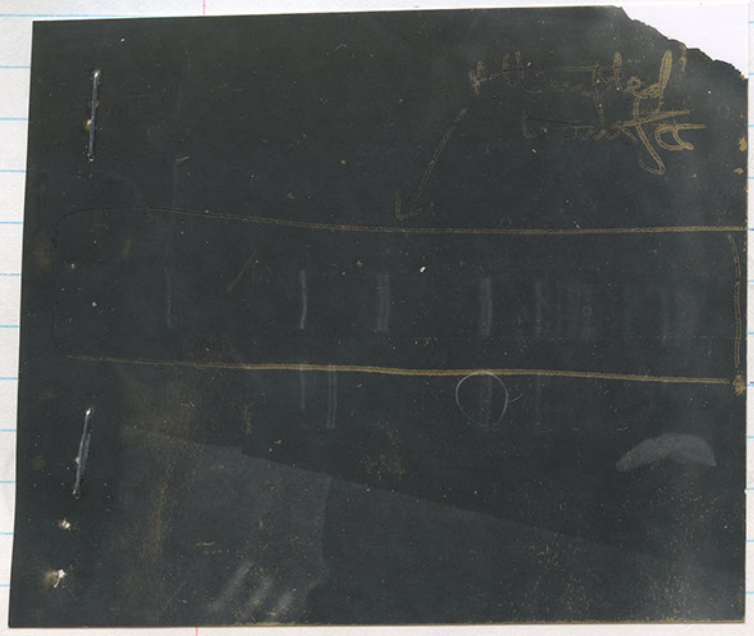
Again Sat 2 pm → Mon 9 am

Mon → Sat noon

Sel
0.25

Good result

Important technical comments



No fete table
transfer by
buyer of - with 48 hr
contract of label

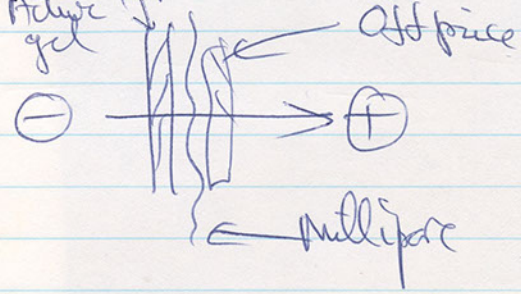
Thurs. March 24th

Small piece gels

Fred suggested electrophoresis out out acrylamide might work. Meanwhile try high conc^n agarose.

Fri, March 25th

Tried an old HaeIII gel (5% acryl) of LAF with ~15 min Sol^n I ~15 min Sol^n II ~15 min el. at ≥1 V/cm in 6xSSC (15 Amps ~2 V/cm in old Lestainer) Active gel



Rinsed. To Dehoff's at 4.30 pm
70°
↓
11.0am

Removed excess overplaced by Used probe P+1 & P-1 from 081
→ to 70° Sat. am

Sun Noon Probe recovered
Rinsed 2x → to 70° with
2xSSC ½% SDS
↓
3pm → 4.30 pm

0133 USED PROBE

2xSSC rinse. Auto. Sun 5pm
↓
Mon 9am

Mon 10am → Wed 10am Negative

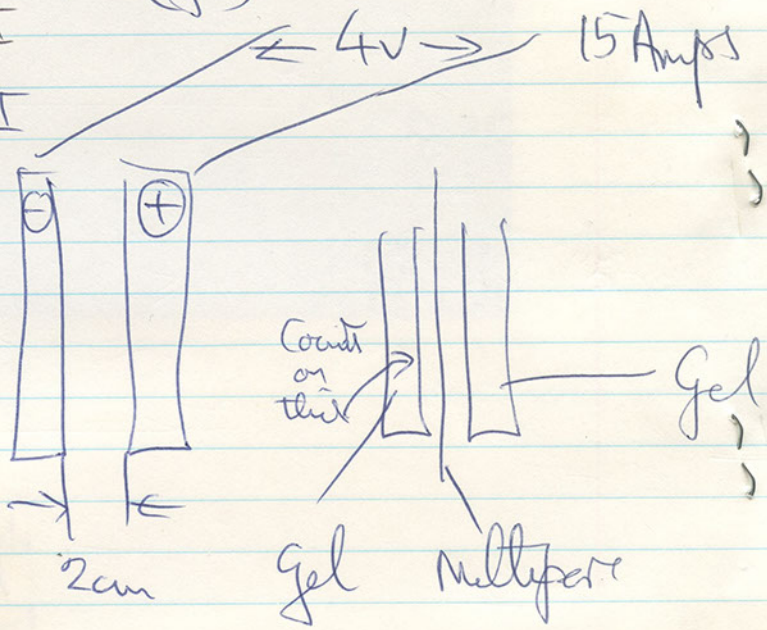
Result shows transfer occurred on both attempts 15 min → 45 min. so if geometry is improved, the method will work!

Mon. March 28th

More serious gel transfer expt.

LAF gel #135 containing ~ 60,000 cpm (of which ~30,000 is in Hha Δlac piece → rest in undigested Δlac).
In outside slot (of 6).

3.40pm to solⁿ I
4.0pm to solⁿ II
4.30pm



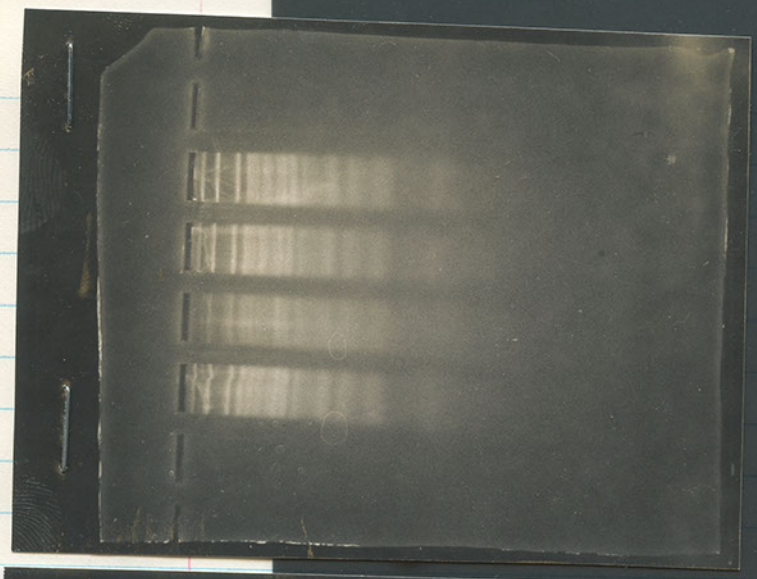
V/cm sh'd be > 1v/cm

4.45pm Counts still in gel
5.05pm tried again (5.35pm pH 9.7)
↓
5.50pm Most of counts still in start gel – must channel current better – none appear to be through – some on Millipore

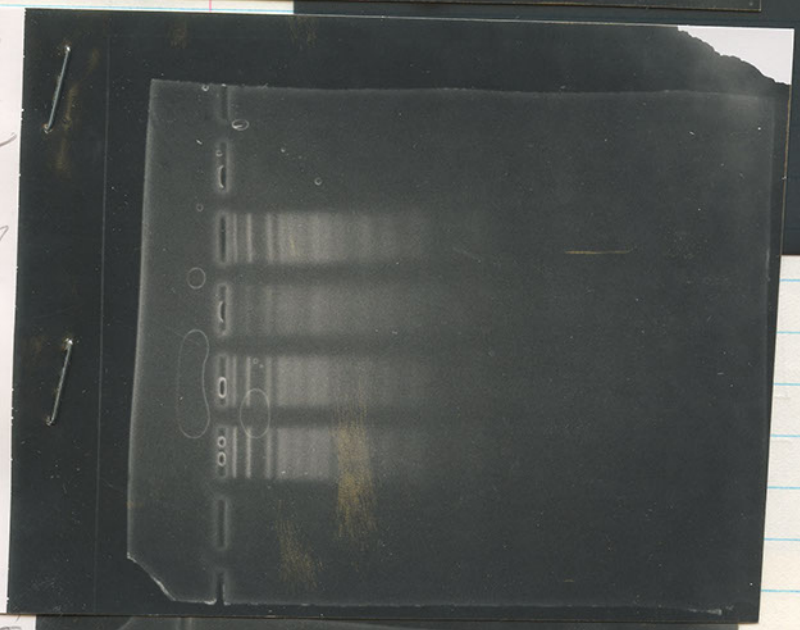
tried to control 6pm → 8am

DNA

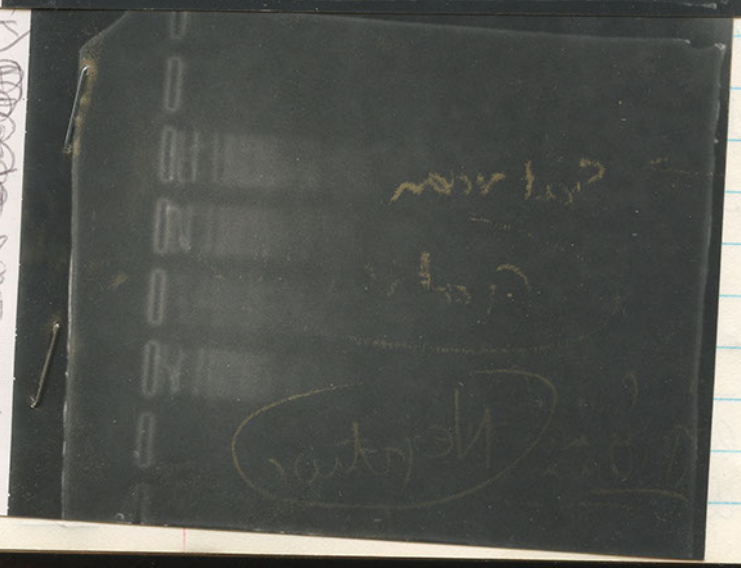
0137 (2)



FRONT) POST TRANSFER



(B) POST TRANSFER



Mon March 28th

HaeIII / BamHI again

On 935 pm
Air cooled.

λAFDB1	Hb4	HaeIII	5% Agarose (autoclaved)
λAFDB1	Hb4	HaeIII/BamHI	4 x H
λAFDB1	ΔLac	HaeIII/BamHI	2.5 V/cm 100 mA
λAFDB1	ΔLac	HaeIII	

At 11.50pm ↓ to 1.0V/cm.

Off 8.30am (slots had fried & current was ~0mA)

Photographed. Sol^n I 8.35am → 9.0 Sol^n II 9.0 → 9.25am

(Top down) Transferred as usual from 9.30 to 4pm
Stained & checked. Transfer incomplete
Denhardt at 70°. Use overnight in future

Hybridized 11.0am filed → recovered probe — Thurs 8.30am
against λB1 (3.5ml)

2 x SSC ½% SDS 70°C 9am → 0.15am 10.20am → 2
→ Rinse

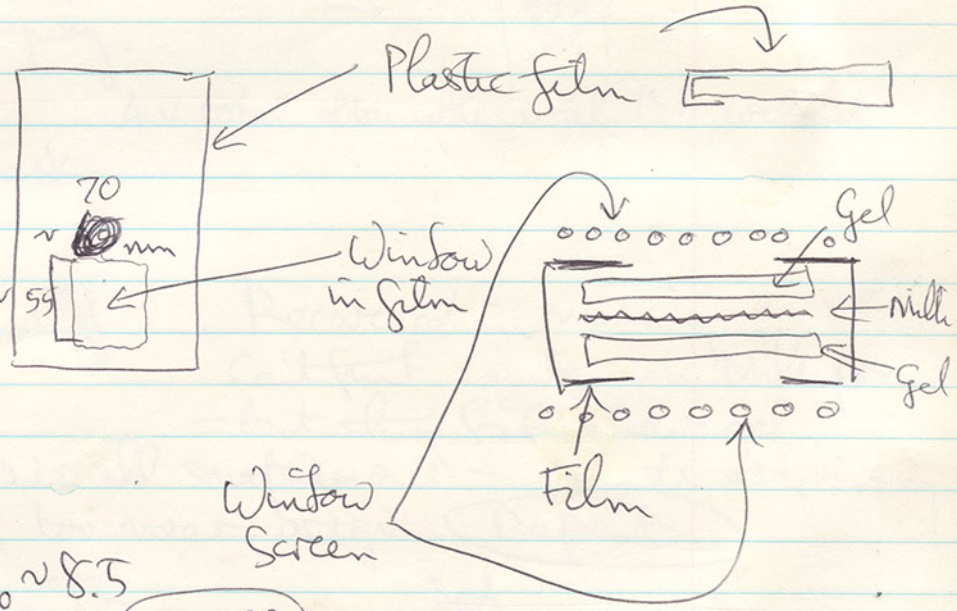
Negative 2 → 4.30 2xSSC Rinse & dry. Autorad 5pm
Sat noon ← 1 Fri 10am ← 1

Wed. April 30th

E. p. transfer

Rough 8% gel run with ~30,000 cpm

12 noon Hha piece ~~from~~ Hb3
to Soln. I 12.20 Soln II



pH_0 ~8.5

12.55 pm On to epi (in 20xSSC) 15A ~6v across - no path around gel (2-3v/cm)

pH_0 ~8.5 pH_{45min} 8.5 (getting warm)

pH_{10min} 8.9 pH_{70min} 8.5 (56°C)

pH_{20min} 8.7 pH_{100} 8.35 (62°C)

pH_{180} 8.4 (65°C) Still ≥2v/cm

fruity smell - not much O_2

No counts on Millipore. Appearance

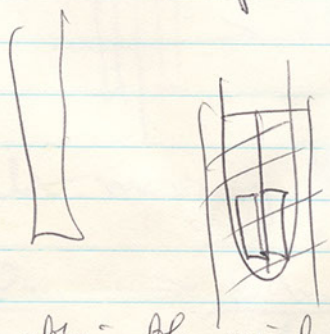
Check after 7 hrs. / suggests gel may have dropped below mark. Counts still in gel. Cleaned up apparatus.

Replaced 20xSSC.

Redenatured DNA in sample

Reset up without mark

— should not alter V/cm anyway.



5.15pm 4V total obtainable with 15A without mask.

↓

9.30pm Off. Recovered in morning

Can't find counts on Millipore

— but they didn't leave gel

This is all satisfactory — counts are, in any case, too near origin. Repeat.

10.30am Th → Sat 12noon

Autorad

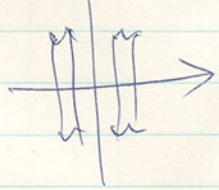
Negative

Thurs. March 31st

~~Lost~~ latest gel ep

Sec. I ~15 min Sec. II ~15 min

6pm ~4V/cm



12 midnight

Still no counts moving! Crazy

Autorad 12.20am → 10am

Nothing!

Negative

Donna's Mackerel — 495 base pairs = 1%λ

2.6
3.7
5.4
6.8 (rt. end)
9.1
9.8
11.8
12.2
14.0
15.4
(17.7) partial see a very high conc.
21.3 ← ? absent
(25.8) partial " " " " "
44
51 fusion (44 + 6.8)

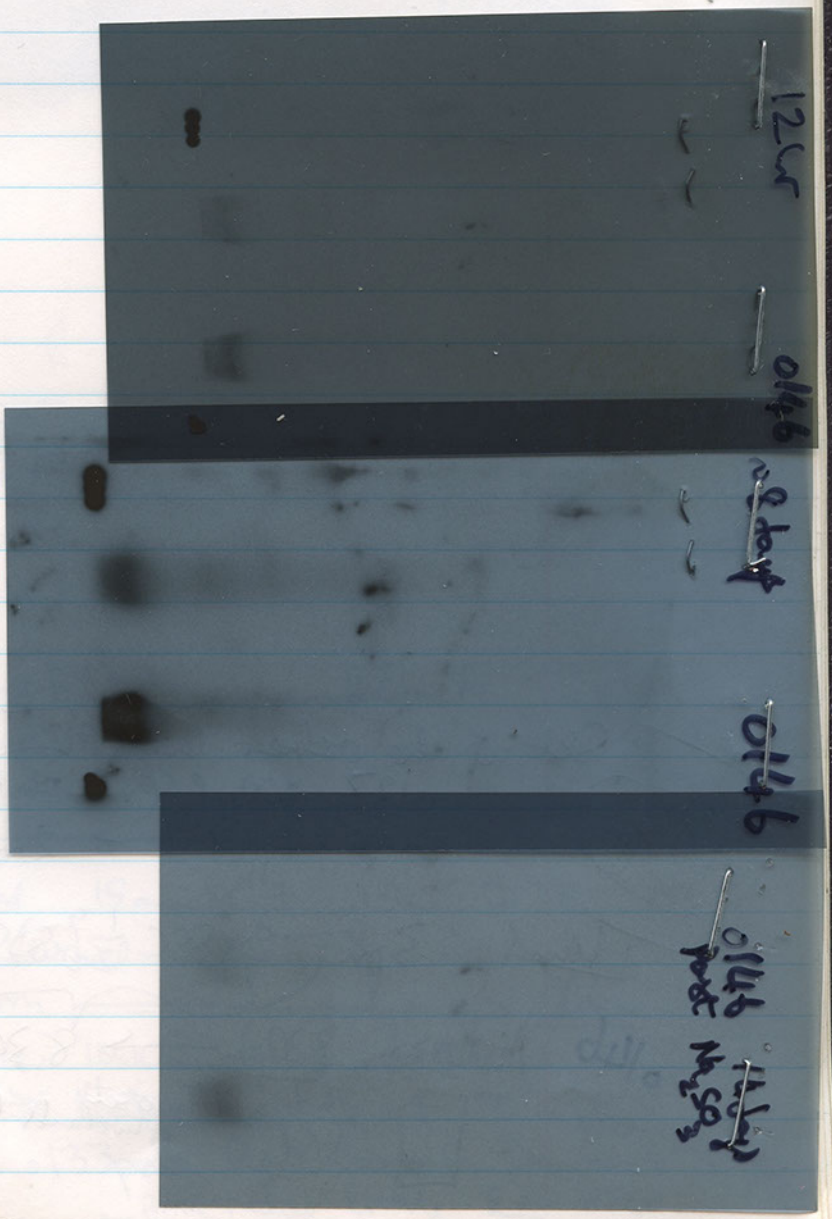
60 KKI Left end
(65) see if had sample fusion (44 + 21)
(67) " " " " " (60 + 6.8)
80 fusion (60 + 21.3)
100

Fri. April 1st

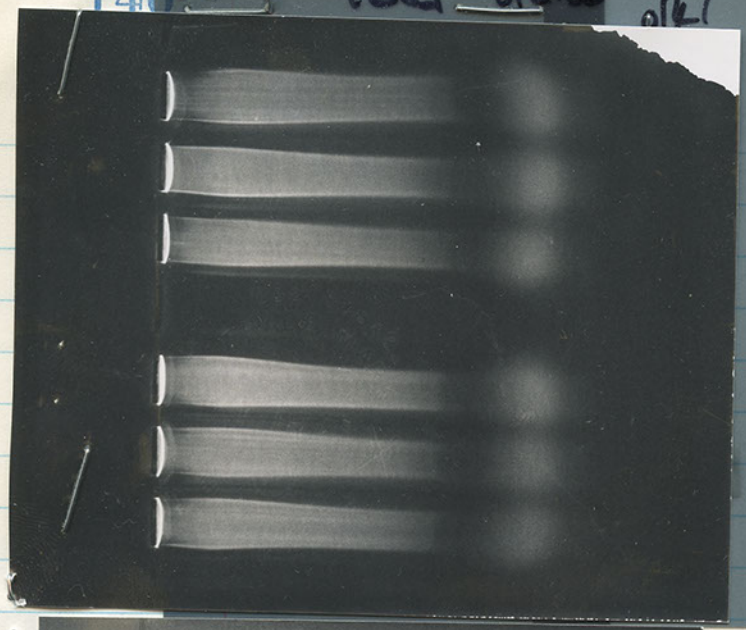
Dauna Mix

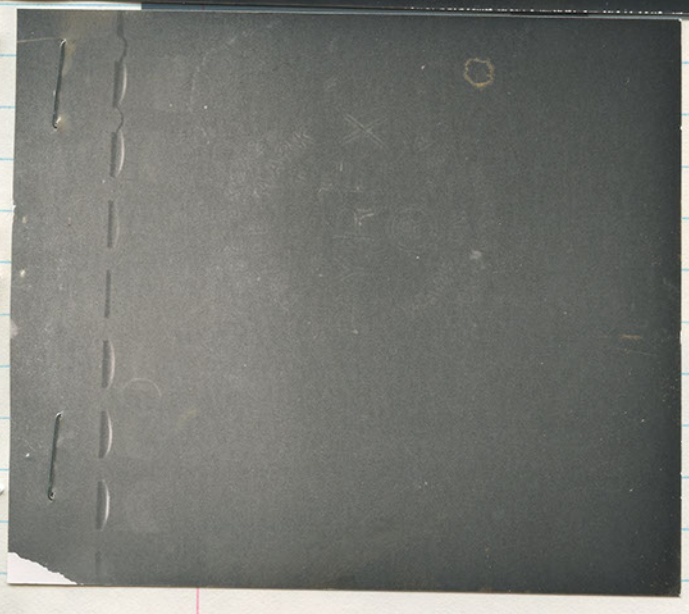
Marker Mix 0145 Conc'd mix

Marker 0145 diluted 1:10 with DNA buffer



12hr 0147 0127 14days ~8days





Result with λ probe shows two faint bands on 12 hr. ~~[illegible]~~ ~~[illegible]~~ – got two pieces muddled

Wed Apr 11th Set up the other half [ ] with Hp'h probe 0127 200 µl (now ≅ 750,000 cpm) in 200 µl 2X annealing sol'n → 2 ml 1X

~3pm

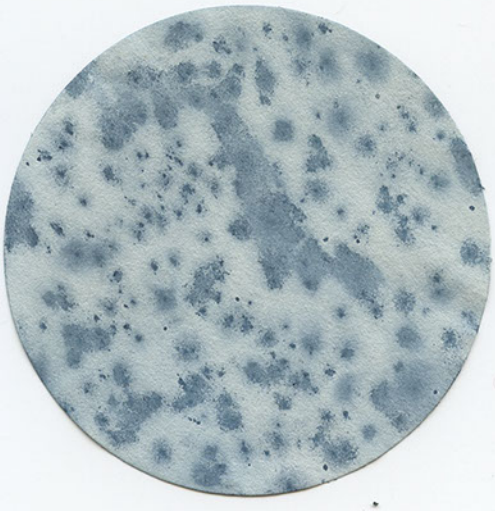
Thurs 3pm 2xSSC ½% SDS → ~4.15 → 5:30 → 8:0

0146 Autorad. 8.30pm → 8.30am

Two sets of two bands

← slots

Fri April 9th → Sat Apr 10th



See back on 145

Did not work — too much bkgd.

Sat April 16th
o146 piece soaked in 0.4M Na_2SO_3
0.2M K_2CO_3
NaOH to pH 11.9
Soak ~5 min. Rinsed in 2xSSC & dried
Autorad.

Sunday April 3rd

Background test

Sat April 16th

Sat 16th → Sat 30th

100µl of 45 Bulk Mouse R1 digest (last of it) to remainder (~10µl) of of 51 10xR1 to give
~ 110µl o147 2x Mouse R1

~55µl

55µl + 1µl o145 Biotinated Marker mix

Hi o147

Sam

On 12.20pm Off 4pm

o147 2x Mouse R1	0.7% Agarose
o147 2x Mouse R1 + λ	4xH
o147 2x Mouse R1	~2.5 V/cm
o147 2x Mouse R1 + λ	100mA
o147 2x Mouse R1	
o147 2x Mouse R1+λ	

Auto 8pm (doesn't look good)

12 h

5.30

Fx

Stained. Photographed. 15 min Sol^n I 15 min Sol^n II

Transferred as usual

5pm → 9am

3.10pm

80° under vac. 10.10am → 12.30

½ 2xSSC

~ 12.30pm → In Denhardt's at 70° 10.30pm

2pm λ

½ off & plus o133probe Rest back to Denhardt's

— both rather little 2pm Fraze

Grunstein – Hogness

9cm Millipore .45μ

boil 3 x 1min in H_2O

autoclave between blotters in petri dish 10' dry in autoclave 10'

place millipore on ~~pt petri~~ ~~medium~~ agar in petri dish no bubbles

replica plate onto millipore filter and grow colonies

The Procedure

1) Place filter gently on a blotter moist with .5N NaOH 7min no air bubbles

2) Place filter gently on a blotter moist with 1m Tris 7.5 2 min

3) repeat step 2

4) Place filter on a blotter moist with .5M Tris 7.5 1.5M NaCl 5-10 min

5) Place filter on filter separates and dry with hand vaccuum 2 min

KHM

optional → 6) ~~2mg/ml~~ Soak filter in 2mg/ml predigested Pronase 1XSSC 2 hrs 37C +5' 65°
15 min dry with vacuum on apparatus

7) Wash filter on filter apparatus with ~100ml 95% EtOH

optional → 8) Wash filter on filter apparatus with ~100ml chloroform

9) Dry with vacuum on apparatus

10) Wash filter with .3M NaCl on vacuum apparatus

11) Dry 80° in vacuo 3 hrs to overnight and handle as any nitrocellulose paper

hybridization

We hybridize in
50% formamide deionized by 2 hrs stirring in amberlite and filter
4 X SSC
20 mM Tris 8.3
.5% SDS

(normally 37°C (rDNA))

for fibrom mRNA 50°C

Grunstein – Hogness

9cm Millipore .45μ

boil 3 x 1min in H_2O in petri dish

autoclave between blotters 10' dry in autoclave 10'

place millipore on ~~pt petri~~ ~~medium~~ agar in petri dish no bubbles

replica plate onto millipore filter and grow colonies

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1) Place filter gently on a blotter moist with .5N NaOH 7min no air bubbles

2) Place filter gently on a blotter moist with 1m Tris 7.5 2 min

3) repeat step 2

4) Place filter on a blotter moist with .5M Tris 7.5 1.5M NaCl 5-10 min

5) Place filter on filter separates and dry with hard vaccuum 2 min

Cos/Prestidge modification of the Grunstein filter screening procedure

(1) With toothpicks stab the colonies to be tested in an ordered array on an L plate (the master plate) and grow overnight at 37°C. A stainless steel template is helpful.

(2) Replicate up to 150 colonies the master to a nitrocellulose filter on an L plate and grow overnight at 37°C. The filter will remain on the surface of the agar while you replicate and the cells will grow normally on the filter.

Store the master plate at 4°C.

We punch our own filters from 293mm diameter HAWP Millipore filters. These are boiled three times in water to remove detergent and then autoclaved (layered between filter paper) for 5 min. (fast exhaust) to sterilise them. The filters shrink a large and variable amount under prolonged autoclaving so it is important to keep to this time. Millipore supply a 90mm HAWP filter which is too large to fit into a standard petri dish (88mm. internal diameter). I have been told that these shrink sufficiently during autoclaving to fit into a petri dish but we have not tried this.

The following steps are performed by placing the filter on blotting paper saturated with the appropriate salts.

(3) Place filter on 0.5N NaOH for 7min.

(4) Transfer to 1.0M Tris/HCl pH7.4 for 2min.

(5) Transfer to a second 1.0M Tris/HCl pH7.4 for 2min.

(6) Transfer to 1.5MNaCl, 0.5MTris/HCl pH7.4 for 4min.

(7) Transfer to a filter block and suck hard for 1min.

(8) Wash with 2x30ml. EtOH and suck dry.

(9) Bake under vacuum at 80°C for 2hrs.

(10) Moisten the filter with the appropriate ^{32}P labelled RNA in 50% formamide 5xssc. and incubate under oil for about 12 hrs. at 42°C. It takes about 0.7ml to moisten a filter. (on mission bottom)

We use $>5\times10^5$ cpm. per filter where possible. For a discussion of this hybridisation step see Grunstein and Hogness PNAS in press.

(11) Wash the filter as follows:

- 2x30min. in 50% formamide 5xssc. at 42°C
- 2x30min. in 2xssc. at room temperature
- 1x30min. in 10μg./ml. RNAse in 2xssc. at RT
- 2x30min. in 2xssc. at RT

2x30min

50 mls

5% 8.5x SSC
2M PB

5% formamide

5x SSC

ideal for pBR 322

Pat

May 20, 1976

Pat,

I'll try to describe the procedure completely. We don't have a protocol annotations written up yet, but I'll send you one when we do. We're ng with ¢¢ bugs growing in individual wells in Microtiter plates so our rements are different then yours. To grow up colonies on filters for dization, we use cookie sheets with fitted aluminum lids. These are d with hard agar with tetracycline and 6 4½" squares of Whatman 3MM are placed on the surface and allowed to wet thru. Then we replicate 12x12 array of bugs on to the surface of the 3MM squares with a 144 'bed of nails' replicator. The bugs are grown overnight at 37°. ria growing over tetracycline containing medium become intensely escent so growth is readily assayed by looking at the filters with UV . (By the way to prepare the filters we simply cut 4½" squares from MM paper and autoclave them. You can number them with a Sharpie marking efore autoclaving and the label will survive all the subsequent procadures. er growth of bacteria immediately over the marking is inhibited. If you r too near the edge, you run the risk of loosing the marking due to atic amputation during batch workup of the filters, so you may want to each filter twice if you do this.) To work up the filters we simply plop into a plastic dish pan with 0.5 N NaOH and wash them gently for about inutes and then change the old stuff with fresh 0.5 N NaOH for another inutes. Then we wash the filters twice with 0.5 M Tris for 2.5 minutes pH 7.4 Then we wash twice with 1 x SSC, again for 2.5 minutes each. Then sh twice with 95% ethanol for 2.5 minutes each and allow the filters to ry. Hybridization is done at 37° overnight or longer in 50% formamide, . We try to have 100,000 CPM/ml of pure probe. Then autoradiography few days gives black spots. After hybridization we wash the filter times with 2XSSC and then RNase at 37° for 1 hr with 20 micrograms/ml eatic RNase. If high background is a real problem, you can wash the rs in Waltham tap water or 0.01 x SSC. This reduces the level of dization somewhat. But we really haven't had problems with background, usually just wash the filters with 2 X SSC. It frequently takes a f washing; we usually monitor the supernatant by Cherenkov counting. ilters are usually still hot by monitor (Geiger tube) (not very hot), ive lovely radioautograms. Be gentle with the filters, they're only . If you see hunks of filters floating around during the washes, slow Air dry when done washing and autoradiograph.

Miscellaneous: It takes about 6 ml to wet our 4½" squares of 3 MM. We dize in plastic sandwich boxes or taller freezer containers. The lab number is 617-647-2434 if you have any questions. I'm also sending atest poop sheet from Stanford on techniques for nitrocellulose filters: ve simplified denaturation and washing of the filters by simply placing ilters on paper soaked with the different reagents. Igor introduced us e use of paper. He noticed that when colonies are lost from the nitrocellulose r during washing, there wasn't hybridization. Reasoning that a 'DNA print' ot the source of hybridization, he tried paper filters since the bugs grow down into the paper and thus not be lost during processing.

Sincerely,

Ralph

I'll be out of town May 21 → 28

Monday - April 4th

Hogness re-visited

70mm filter papers available from stores are:—

1, 2, 40, 42 & 50

~10 of each washed on Buchner with hot d.d.w. Autoclaved.

Tues. April 5th

Decided to test with a λ probe on Y mel (± λ) — this is more stringent for #'s of copies but less since λ is so big.

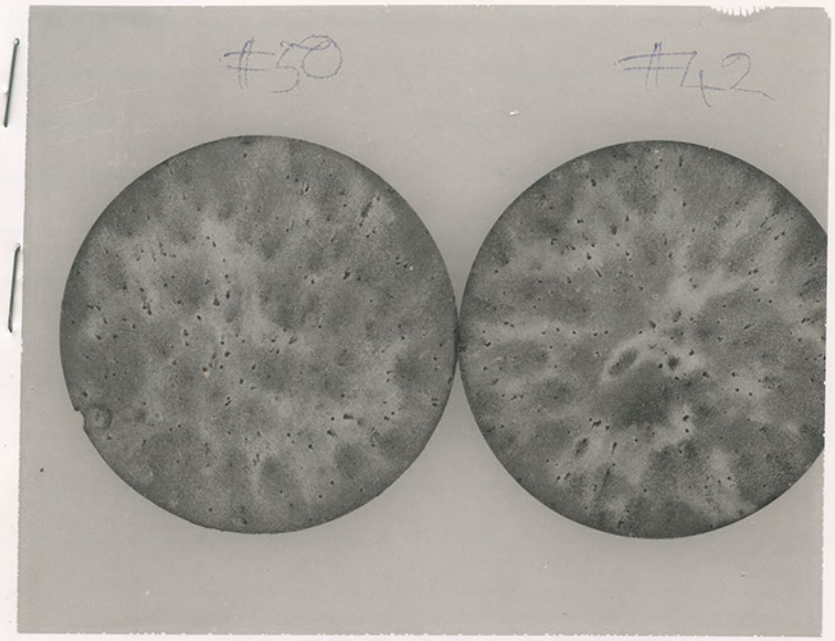
Sat.d cultures available — assume ~ 1×10^9/ml
— took 1ml of KH802 (3/29) + 0.1ml KH802(λ) (4/4)
Diluted to 10^{-5} (i.e. ~10^4/ml) → 10^{-6} (~10^3/ml)
Spread 0.1ml on the papers over NZ plates — papers pre wet in NZ

#1, #2 | More fragile — too full of liquid
#40, #42 | Tougher — nicer water content
#50 | too unabsorbant — but better than 1 & 2

Longer sit before spreading
10^{-5} gave same conclusion.

To 37° 3.45pm

12pm
teed. growing OK

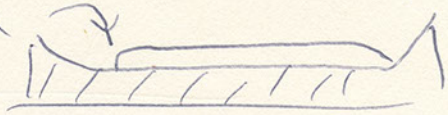

#50
#42

Wed pm looks as if the ~~thinner~~ papers give bigger colonies more quickly.

Thurs am — heavy peri filter paper growth

Stained colonies (AB, dilute, in 50/50/10 with uptake). Clear that radial transport has occurred — presumably by evaporation.

Repeat & seal plates — also put papers down

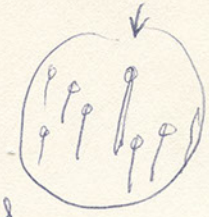


— not up

Fri am Checked re-incubation of stripped dishes — clear that bacteria are through OK but removal smears them

e.g. X1776 #42 5.1

X1776 #40 5.1



So will have to pick colonies or print a replica. Try this on next batch.