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DNA Gel Binding Assay, Procedure (1 of 3) Protein Related

Make 6% gel for Gel binding assay as described on card.

Pre-electrophorese while circulating buffer with the pump for 30 min. or soak gel 2h to overnight in electrophoresis buffer and slice off any gel projecting beyond edge of glass plate.

Store DNA in TE + 50 mM KCl (with no salt you often get extra ss bands). If there is any sign of aggregation, heat the DNA to 65□ 5 min first. If using digested plasmid DNA, 0.5 µg is good.

If necessary, dilute C through 1 x GBA buffer. For dilution, add C to tube, and slowly add the 1 x GBA with mixing. The reverse order kills many oligomeric proteins including C and RNAP. Typical assay uses 0.1, 0.3, and 1 µl of C at 0.05 mg/ml.

Bring tubes to 37□ and add C for 5 min.

DNA Gel Binding Assay, Procedure (2) Protein Related

C bound to DNA is very shear sensitive, stir hardly at all.

Add about 10% volume of the GBA dye most gently. Too much glycerol here can reduce binding.

Add approximately 1 μl of 1% bromophenol blue and load on the gel.

Alternatively, add 5 μl of 0.2% bromophenol blue in 20% glycerol. Either method works.

Samples can be loaded with the circulation pump on.

Electrophorese

Minigels	20 to 25 ma	(50v)	45 min to 90 min
Maxigels	40 to 50 ma	(150v)	45 min to 90 min

Turn pump on after samples have entered gel.

Keep temperature of the buffer at or below 20E.

If staining, stain in 1 μg/ml ethidium bromide for more than 45 min and photograph.

Dry if autoradiographing, dry about an hour, and expose on phosphorimager

screen (leave a layer of Saran wrap between gel and screen.) or autogradiograph.

Bands that look smeared often are not. They may be tilted within the gel either forward or backward. Running too fast, (too hot) seems to make them tilt backwards. To cure bands which tilt forward, just at the time of loading samples, add NaCl to make several millimolar in the electrophoresis buffer.

GBA Electrophoresis Buffer		2 x GBA Buffer	
10 mM Tris-OAc pH 7.4		10 ml 1 M Tris-OAc pH 7.4	3 ml
1 mM KEDTA pH 7.0		5 ml 0.2 M KEDTA pH 7.0	1.5 ml
		485 ml H_2O	145.5

6% GBA Gels	25ml	50ml	75ml	100ml	150ml
20% Acrylamide/0.33% MBA	7.5	15	22.5	30	45
2 x GBA Elect. Buffer	12.5	25	37.5	50	75
H_2O	5	10	15	20	35
Degas 2-10 min					
Amm. Persul. (50 mg/0.5ml)	0.1	0.15	0.2	0.3	0.45
TEMED	10µl	15µl	20µl	30µl	45µl

Another test of indenting to see if it still works here and on the next card.

DNA Gel Binding Assay, Recipes (2) Protein Related

GBA Buffer	5 x GBA Stock (5ml total volume)
10 mM Tris	0.25 ml 1 M Tris-OAc pH 7.4
1 mM KEDTA	0.1 ml 0.25 M KEDTA pH 7.0
50 mM KCl	0.625 ml 2 M KCl
1 mM DTT	250 µl 0.1 M DTT (7.7 mg / 0.5 ml)
5 % glycerol	1.25 ml glycerol
50 mM L-arabinose	0.95 ml 20% L-arabinose
	1.58 ml d H_2O
If working with dilute C	NP40 to conc. of 0.05%

Do not autoclave the arabinose and use good water. This is a test of indenting

GalK Assay (1 of 6) Protein Related

Grow cells with plasmid overnight in YT amp to stationary.

Dilute cells at least 200-fold into minimal medium and grow to an OD 550 not over 0.3.

Casamino acids at 0.5% may be added to the medium, but complications from catabolite repression may occur.

The safer method is to use sterile 125 ml flask with 20 ml M10 B1, glycerol, and any other requirements plus 20 μg/ml ampicillin.

For convenience since the cells can take 6 hours to grow up, add the cells from YT to flask and set timer to turn on air incubator at 4 A.M. at 37E.

Alternatively, cells can be grown in 4 ml of medium in the tube roller, but again, complications from inadequate aeration may occur. In the tube roller and with casamino acids present, cells will be ready in about three hours.

GalK Assay (2) Protein Related

Add 100 to 1000 µl of cells to eppendorf tubes containing sufficient chloamphenicol (50 mg/ml in ethanol) to give a final concentration of 200 µg/ml and centrifuge 5 min. Have tubes on ice and keep tubes on ice for all following steps unless otherwise noted.

Resuspend cells in 100 to 1000 µl of cold M10 medium + 200 µg/ml chloramphenicol (volumes will depend on enzyme levels).

Add 1/25 vol. of mix D and 16 µl tolune per ml of cells used. Vortex 30 sec and and incubate at 35 -37E 10 min to remove toluene

Due to fluctuations in the assay, perform the assay in triplicate for each sample. To avoid insanity, use a microtiter tray.

Add 20 µl of the toluenized cells to wells containing 20 µl Mix A and 50 µl Mix B. Be sure to include a no cells blank.

Warm to 32E and add 10 µl of Mix C (^{14}C-gal)

Incubate 30 to 60 min, recording exact time.

Stop reaction by addition of 10 µl 0.25 M EDTA

Slowly add 75 µl of the reaction mix to the center of a 2.5 cm diameter DE81 filter on a Millipore filter apparatus

Wash with 20 ml H_2O releasing approximately 5 ml at a time from the pipette and remove the glass chimney for the final 5 ml of wash. (Blanks should have less than 100 cpm).

Prepare two sample filter papers with 25 µl of reaction mix and do not rinse.

Measure radioactivity on filters. With ACS counting fluid, filters need not be dry. They should be uniformly damp however for reproducibility.

Units are nanomoles gal-PO_4/min/ml of OD_{650} cells.

100 nanomoles ofgalactose are added per assay. Since we count 75 µl of the 100 µl reaction mix, the amount of galP produced is proportional to 100/75 times the cts in a sample. Since the unwashed blanks contain 25µl of the 100, the amount of gal in the assay is 4 x

the cts in the unwashed sample. Since the assay is units per 1000 µl of unconcentrated cells, and since 20 µl are used, the amount of phosphate converted must be multiplied by 1000/20 = 50. Overall, these factors yield a converstion factor of 100 x 100/75 x 1/4 x 50 = 1667. Note, until 5/86 we incorrectly used a factor of 3968.

U ((CPM - blank) x 1667) / (OD_{550} x concentration factor x incubation time x average of unwashed blank).

Note that cells may have to be diluted or concentrated for the assay and that the assay is linear up to about 15% conversion of label to galactose phosphate.

GalK Assay (5) Protein Related

Mix A

5 mM DTT	7.7 mg
16 mM NaF	6.7 mg
H_2O	10 ml

Mix B

8 mM $MgCl_2$	80 µl 1 M
200 mM Tric-HCl pH 7.9	1 ml 2 M
3.2 mM ATP	17 mg or 320 µl of 100 mM
H_2O	8.9 ml or 8.6

Mix D

100 mM EDTA	4 ml 250 mM
100 mM DTT	154 mg
50 mM Tris-HCl pH 8.0	0.5 ml 1 M
H_2O	5.5 ml

Mix C

D-(1-^{14}C)galactose at 40-60 mCi per micromole in 1-5 ml. Dry 60 µl, add 0.01 millimole of cold galactose (9 µl of 20%), and bring to 1 ml with H_2O. Filter twice through DE81 filters in a Swinnex filter to reduce the blank background. This proceedure gives 100 nanomoles galactose per assay.

Conversion of label to galactose phosphate must be significantly more than the background levels of about 50 cpm. Generally, the following is useful.

	Recommended Conc. Factor	GalK Units
WT p_{BAD} fully induced	0.1	150-200
repressed	10	2-5
C^-	10	1-2
p_{BAD} fully repression minus	2	8-10

BioRad Protein Assay — Protein Related

800 - x µl H_2O or protein buffer
x µl protein solution
200 µl BioRad assay mix
Let sit 5 min and read OD.
Zero the spectrophotometer with a blank containing the same components minus protein.
Make a standard curve using BSA.
Protein concentration in µg/ml in the solution . 20 x 800/x H OD_{595}
Note, response of assay declines with age of assay mix. Recalibrate each six months.

1. Break 100 gm of -70E cells with prechilled large mortar and pestle.
2. Add 300 ml of fresh grinding buffer and pieces of frozen cells to chilled blender. Blend at high speed for 3 min and incubate 20' at 4E.

Grinding Buffer

50 mM Tris-HCl pH 7.9	7.5 ml 2M Tris-HCl pH 7.9
5% Glycerol	15 ml 100% glycerol(heat to pour)
0.2 mM NaEDTA	0.3 ml 0.2 M NaEDTA pH 7.5-8.0 .12ml 0.5 M
0.1 mM DTT	4.5 mg dithiothreitol*
1 mM Mercaptoethanol	21 µl 14.6 M 2-mercaptoethanol*
232 mM NaCl	4.06 g NaCl
Lysozyme	39 mg lysozyme*
0.13 mM PMSF	6.9 mg phenylmethylsulfonylfluoride (disolved in 0.5 ml EtOH)*
100 mM L-arabinose	22.5 ml 20% arabinose
	to 300 ml with chilled dd H_2O

*-Add immediately before use

3. Add 6.25 ml 4% Na^+-deoxycholate, blend 30 sec. Incubate 1 1/2 hr at 4E. Cells lyse and solution becomes very viscous.

4. Add 400 ml TGED, blend at high speed for 30 sec, spin down in large centrifuge bottles, 12 K, 20 min. Prechill GSA rotor.

Collect supernatant (approx. 700 ml)

TGED

10 mM Tris-HCl pH 7.9	2 ml 2 M Tris-HCl pH 7.9
5% Glycerol	20 ml 100% glycerol
0.1 mM NaEDTA	0.2 ml 0.2 M NaEDTA pH 7.9, 80 µl 9.5 M
100 mM L-arabinose	30 ml 20% L-arabinose
232 mM NaCl	5.4 g NaCl
0.1 mM DTT	6 mg DTT
	To 400 ml with dd H_2O

10% Polymin-P

To 15 ml Polymin-P (21 g) (polyethylenimine), add 50 ml dd H_2O.
Adjust to pH 7.9 with 2 M HCl or conc. HCl.
Bring to 150 ml with dd H_2O.
Spin out debris at 12 K, 40 min.

5. Add 10% Polymin-P over a 60 minute interval using P3 peristaltic pump to a final concentration of 0.6%. If volume is V mls, add 0.6V/9.4 mls.
Let stir an additional 10 min. Do on ice.
Spin 8 K 30-50 min.
Collect supernatant and add $ZnSO_4$ to make 0.5 mM.
6. Potassium phosphate precipitate the supernatant.
Add KPO_4 mix, 0.254 g phosphate per ml solution. Add over a 90 min interval while rapidly stirring and keeping below 4E. Let each amount added dissolve before adding the next portion. After adding the final amount, let stir 10 min.

K-Phosphate Mix

14 g KH_2PO_4 (beware waters of hydration). Grind this first, then add
186 g K_2HPO_4
Grind until homogeneous

7. Spin down 8K 30 min. Save pellet. As supernatant will still be cloudy, spin again 9K 30 min, save pellet. Resuspend both pellets in a total of 20 ml of 0E D buffer. Dialyze 2 hr, 1 hr each time against 1 l D buffer. Use boiled Number 20 tubing and rocking dialyzer in the cold room. Keep an air bubble in the sack to stir the solution.

D Buffer, 0.08 M Phosphate

10% Glycerol (v/v)	126 g glycerol, or 100 ml 100%
(20% glycerol is used	20 ml 2.5 M K_2HPO_4
for storage of AraC)	0.9 ml 2.5 M H_3PO_4
	or, 8.71 g K_2HPO_4
	0.9 ml 2.5 M H_3PO_4
10 mM L-arabinose	7.5 ml 20% arabinose
1 mM KEDTA	5 ml 0.2 M KEDTA pH 7.0
0.1 mM DTT	15 mg DTT
0.5 mM $ZnSO_4$	5 ml 0.1 M $ZnSO_4$
	To 1 liter with dH_2O

Make 3 l of 0.05 M D as above.

For FPLC also make 1 l 0.05 M phosphate minus DTT and minus glycerol (A) and 1 l of 0.5 (10 x the phosphate components) also minus DTT and glycerol (B). Filter these last two with the FPLC filtration glassware with 0.45 μ (HA) filters.

8. Spin down 12 K 20 min. Resuspend pellet in 150 ml D buffer (with glycerol). Crush by passing through fine homogenizer. Stir slowly overnight in cold room. Use spacer (petri plate) between stirrer and flask.

9. Spin down 12 K 20 min in Sorval 50 ml tubes. Save supernatant which should be about 0.5 mg/ml 80% pure araC protein. 1 mg/ml = OD_{280} 1.43.

Using FPLC Phosphocellulose Column

10. Set valves for the phosphocellulose column and monitor for 280 nm, and UV monitor for 0.50 (OD of 0.5 is full scale), Max. Pressure 0.5 MPa.

11. Degass the filtered 0.05 and 0.5 M phosphate buffers, 10 min in suction flask with a stirring bar.

12. Load buffers into FPLC pumps, A = 0.05, B = 0.5, by running WASH. Make sure valve V7 is in wash position. Choose manual control, step forward until WASH appears. Enter 1.1 (pumps A and B), Do store to enter and begin. The wash procedure takes about 5 min.

13. Move valve V7 back to load, and run 0% B over phosphocellulose column until baseline level is reached, approx. 20 min. Choose manual control, 0% B, 2 ml/min, .5 cm/ml, no portset. End to quit.

14. Run crude C prep into largest tubing of peristaltic pump P-3. Remove all air in lines and connect output of peristaltic pump P-3 to port 2 of valve V-7 using the black connector on one of the large tubes.

15. Move lever to inject. A tube connects the bottom of PC column, to port 4 of valve V-8. Disconnect from V-8 so flow through of column can be collected in a beaker.

16. Load crude AraC onto column at 12 mg/hr, typically 5 on speed scale of pump P-3.

17. When C has loaded, stop P-3 and switch lever on V-7 to load. Make sure no air bubbles get into column. Reconnect sample loop to port 2 of V-7. Reconnect bottom of column to port 4 of valve V-8.

18. Set fraction collector to collect 5 ml fractions by pressing fraction size button (7), pressing 5, then pressing Store-Return. Load collector with 40 tubes.

19. Run elution program, currently Bank 1 Method 5. Method File, 1; Do Store; Exit; 5; Do Store.

Time/vol	
0	1 ml/min
0	0% B
0	0.1 cm/ml
0	portset 6.1
75	0% B
150	100% B
	End method

AraC elutes as a peak near full scale at around 50% B.

Measure OD_{280}, add glycerol to 20%, mix, store at 4E.

20. When finished, pour 200 ml D buffer A into 200 ml flask and add 400 µl 1 M NaN_3. Place intake tube and endfilter from pump A in flask.
21. With valve V7 in wash position, wash the azide buffer into pump A, Manual; Wash; 1.0; Do Store.
22. Pass the azide flush over column for about 90 min. Valve V7 on load position, Manual, 0% B Do Store; 1 ml/min. Do Store; .5 cm/ml Do Store; until OD reaches baseline, about 90 min.

23. Return FPLC to oligo mode
    Wash oligo FPLC buffers into pumps (V7 on Wash)
    Max Pres. 4 Mpa
    Reset valves for MonoQ column (position 3)
    Reset monitor to 254 nm, Range to 2.0
    Change fraction size to 1 ml (Frac. size 1, Store-Return)
    Method file 2, Do Store, Exit

Old procedure for phosphocellulcose column

Load on a 3.5 cm diameter by 12 cm long, 75 ml phosphocellulose column equilibrated in D.

Load at 12 mg/hr (Yes, milligrams/hour). Collect 10 ml fractions on Buchler LC100 fraction collector during loading. Wash with 75 ml D buffer at 10 ml/hr, 1.65 on pump. Collect 10 ml fractions every 60 min. Elute with a 500 ml total phosphate gradient 0.08 to 0.8 M in D at 10 ml/hr collecting 10 ml fractions.

10. C protein should elute in the major peak, at about 0.45 M phosphate. Occasionally it is preceeded by a peak containing almost as much protein.

β-Galactosidase Assay, Eppendorf Tube Protein Related

Grow cells and appropriate control strains in M10 to OD_{550} . 0.9.

Spin down 1.1 ml cells, 2' at full speed.

Resuspend cells in 1.1 ml lac Z-buffer (remember to add β-mercaptanethanol, 2.7 µl/ml, no ONPG.

Add 1 drop 0.1% SDS, 2 drops $CHCl_3$ (H_2O saturate, take bottom layer), vortex 15 sec.

Remove 100 µl to a second tube containg 900 µl Z-buffer, no ONPG (1/10 dil).

Add 200 µl 4 mg/ml ONPG in Z-buffer to each tube and a number of no cells blanks, vortex, and start timer.

Incubate tubes at 30E, and when a tube becomes straw yellow, remove it, and a blank, note the time, and add 0.5 ml 1 M Na_2CO_3 (not autoclaved.)

Spin tubes 5 min. at full speed and read OD_{420}.

β-gal units = (1000 x OD_{420})/(Cell OD_{550} x Incubation time (min) x ml cells used).

Phosphorimager Protein Related

Expose dried gel to phosphor screen (layer of Saran wrap between gel and screen) 2-4 hours for typical ^{32}P experiments or overnight for ^{35}S.

Insert screen into phosphorimager screen side down, push blue handle all the way in, turn it straight down, and close the outer door.

In Windows, start IQ, which is found in the Program Manager.

Under Scan, choose scan sample, enter the file name and click on scan.

After the scan, darkness and contrast as well as color can be adjusted using options available under the view choice.

To quantitate a specific area, select Objects and use the rectangle and integrate.

Transfer your file to your own Zip disc, for you cannot be sure files on the computer remain intact.

Remove screen and erase on the light box, 8 min.

Sensitivity to sigma or AraC is about 0.001 μg. Separate proteins in appropriate SDS gel using the Hoefer apparatus. Identify orientation of gel by cutting off upper right corner.

Electroblotting buffer

25 mM Tris	12.1 gm Trizma base
192 mM Glycine	57.6 gm Glycine
20% Methanol	800 ml Methanol
0.01% SDS	4 ml 10%

Adjust to pH 8.3 with Acetic acid.

Adjust to 4 l with H_2O.

Equilibrate gel in 250 ml electroblotting buffer 30 to 120 min.

Wet transfer membrane (DuPont PVDF) cut to the size of the gel briefly in methanol then transfer to a tray of electroblotting buffer. Use the BioRad electrotransfer apparatus. Also wet in the tray Whatman 3MM paper cut to the size of the gel, the fiber pads, and the transfer

membrane.

Assemble transfer sandwich in the order:

- Black side of holder (cathode side)
- Fiber pad
- Whatman 3MM paper
- Presoaked gel
- PVDF membrane (Remove any air bubbles by rolling a glass pipette over the membrane.)
- Whatman 3MM paper
- Fiber pad
- Other side of holder

Clasp the holder shut and place in the gel running tank filled with electroblotting buffer and containing a small stir bar.

Run at 50 V (lots of current, use BioRad 2.5 A power supply) 4 hours. Keep at room temp. by running tap water or ice water through the cooling coil.

Immunoblotting (1 of 3) Protein Related

TBST

10 mM Tris-Cl	0.48 g Trizma base
150 mM NaCl	3.5 g NaCl
0.05% Tween 20	200 µl

Adjust to pH 8.0 with HCl

Adjust to 400 ml with H_2O.

Blocking solution: 1% Calbiochem fatty acid poor BSA in TBST. Dissolve 300 mg BSA in 30 ml TBST.

Ab solutions:

Add 100 µl monoclonal mouse Anti-AraC Ab to 30 ml TBST.

Add 10 µl Sigma anti-poly histidine Ab to 30 ml TBST. (For His-6 proteins.)

For secondary Ab, Promega alkaline phosphatase conjugated secondary mouse Ab, add 4 µl to 30 ml TBST.

These solutions can be used many times. Store at 4E.

Immunoblotting (2) Protein Related

Float membrane after Western transfer on 30 ml TBST until evenly wet.
Submerge and soak 5 min.
Decant TBST, add blocking solution and gently shake 30 min.
Decant blocking solution, add primary Ab solution, and shake 30 min.
Decant, add fresh TBST, and shake 10 min. Do this twice.
Decant, add secondary Ab solution and shake 30 min.
Decant, add fresh TBST, and shake 10 min. Do this three times.

Alkaline Phosphatase Reaction Buffer

100 mM Tris-HCl pH 9.5	0.36 gm Trizma base
100 mM NaCl	0.18 gm NaCl
5 mM $MgCl_2$	30 mg $MgCl_2$

Adjust to pH 9.5 with HCl
Adjust to 30 mls.
Add 200 µl Promega NBT solution and protect the solution from excessive light.
Add 100 µl Promega BCIP solution and mix.

Incubate the PVDF membrane in the reaction buffer for up to 30 min. When the color development is optimum, stop development by rinsing in dH_20.

FPLC Purification of Oligos (1 of 14) Larger Equipment

Works for oligos 15-60 (maybe larger) long, for 2 μgm to at least 1 mg.

Oligos are to be synthesized with trityls off.

After deprotection, ethanol precipitate and resuspend in 100 μl TE. Take 50 μl plus 950 μl A buffer (7) for FPLC purification. Spin 5 min in microcentrifuge before use to remove particulates.

Use a MonoQ column, HR 5/5 (5mm diameter, 5 cm long) and a sample loop of 500 μl or 1 ml.

Following a power outage, the pumps and recorder parameters may need to be reentered, pump A/B 110, recorder cm/min 200. Under calibration parameters:

Cal-MIN0 ML.1 DL.2 1

Pump CAL AB 110

REC CAL 200

Purification of Oligos (2), General Protocol Larger Equipment

Adjust pumps (3), UV-M (4), recorder (5), and fraction collector (6) to standard settings and programmer to volume mode.

If the machine has previously been used for another application, wash pumps (9) with A and B buffer (7, 8).

If the column has not been used for oligo purification in the past week, or was last used for some other application, run (10) Startup Program (11). This is currently stored in Bank 2, Method 1

Run Purification Program (10, 12, 13). This is currently stored in Bank 2, Method 0. Note that fraction collector starts only at 8 ml.

Column can be stored as is since the acetonitrile will preserve it.

If machine will not be used for more than a few days, wash pumps (9) with filtered (7) water.

Purification of Oligos (3), Notes on the pumps Larger Equipment

Standard settings

Pulse comp button depressed

Flow rate 000

While pressure button is depressed, set pressure limit to 3.5 MPa

If pressure limit has been exceeded, alarm will be sounding and limit lamp on the pump will be on. To resume run, press and hold the set button on pump to release the system to continue and press the alarm reset button on the programmer.

Purification of Oligos (4), Notes on UV Moniter UV-M Larger Equipment

Turn on UV lamp one hour before use.

Standard Settings

AU, absorbance units, for half of a 0.2 μM scale synthesis, set for 2.

Adjust the filter wheel on the filter part of the unit to 254 nM.

To turn lamp off, hold the on-off switch depressed for 3 sec.

Note that the path length of the moniter is 0.5 cm. Therefore a sample that indicates an AU value of 1.0 on the moniter will have an OD of 2.0 on a conventional spectrophotometer with 1 cm path length cuvettes.

When applying more than 100 μgm, the peak of desired oligo will be off scale at 2 AU. This is not a problem since the preceeding peaks of impurities will be on scale and have normal shapes, showing all is OK.

Purification of Oligos (5), Notes on Recorder Larger Equipment

Standard settings, marked with black dots on recorder.

Speed 10, mm/sec, also depress Ext

Short pen: 100 mv full scale, 100x and 1.

Long pen: 1 v full scale, 100x and 10.

Turn on.

Before starting run, zero adjust both pens to 0 on the chart.

The short pen records the OD from the moniter. When set this way, full scale on the recorder is the absorbance shown on the UVM moniter.

The long pen records the per cent B buffer, going from 0% to 100% full scale.

Chart speed is regulated in part by the program and in part by the recorder settings. In order that chart speed be as you program it, use the above settings.

Standard Settings

Mode 2 (volume)

Volume 1.0 ml

Fill with 12 x 75 mm tubes. Turn collector table so tube 1 is under drop outlet. To position arm, lift up and allow spring to bring to contact with the tubes.

To set mode, be sure collector is on. Switch is in back. Should read "End". If not, press Store/return. Press "1" the number "2" should appear and blink. This indicates the collector is set to collect on the basis of volume. Press Store/return. If 2 did not appear, enter 2, and press Store/return. Display then shows AEnd@.

To set the volume to be collected, press 7, The volume which was previously set should appear and blink. If you want to continue with this value, Press Store/return. If you want a different value, enter the value desired and press Store/return. Dislay shows AEnd@.

Purification of Oligos (7), Water and Buffers Larger Equipment

To preserve the expensive, ($500-$1000) columns, rinse water and buffers must be clean and filtered through .22 μ filters. Use 47 mm filters, and the special clean Millipore apparatus which is kept under the FPLC. Pure water may be filtered through MF-Millipore, GSWP, but discard the first few mls of filtrate. "Durapore" filters, GVHP must be used for buffers containing acetonitrile. HPLC grade acetonitrile can be assumed to be free of particles and instead of using GVHP fileters, can be added after filtering the other components. Durapore is not easily wet by water, and you must filter 5 ml of methanol first, then your buffers.

A Buffer

20 mM Tris.HCl pH 8.0	20 ml 1 M Tris.HCl pH 8.0
20% acetonitrile	200 ml HPLC grade acetonitrile
H_2O	Add to make 1 l

Purification of Oligos (8), Water and Buffers Larger Equipment

A Buffer

20 mM Tris.HCl pH 8.0	20 ml 1 M Tris.HCl pH 8.0
H_2O	780 ml H_2O, Filter and degas (filtering degasses if you let the aspirator suck a few minutes.
20% acetonitrile	200 ml HPLC grade acetonitrile

B Buffer

20 mM Tris.HCl pH 8.0	20 ml 1 M Tris.HCl pH 8.0
1 M Nacl	58 gm NaCl
H_2O	Add to make 800 ml, Filter and degass.
20% acetonitrile	200 ml acetonitrile

This takes about five minutes and is needed only if changing the buffer in the pumps.

Set injection valve to Wash so the wash material bypasses column.

Press Manual button on controller to enter the manual block.

Press Step Forward until you reach Wash A.B.

Enter 1.1 (Pump A is 1 and pump B is .1) and press Do Store to tell to wash both pumps.

To run a program, first get into the correct bank, there are five total, each of which holds up to ten methods or programs. Press Exit, to which Run Method and a blinking 0 should appear. Press Method File. The series of numbers are methods not yet programmed on this bank, and the number blinking at the right is the number of this bank. Enter the bank number you want and press Do Store. Then press Exit. Run Method appears, with 0 binking. You are now in the bank you last selected. If you want to run a method other than 0, enter its number and press Do Store. This starts the machine under control of the program.

Purification of Oligos (11), Program to Clean Column Larger Equipment

Currently in Bank 2, Method 1. Run with injection valve set to inject.

0.00		0% B	
0.00	ml/min	1.0	Flow rate
0.00	cm/ml	0.5	Chart speed
2.50		0% B	
2.50		100% B	A step to knock anything off. Note that this goes from 0% to 100 % in zero time.
7.50		100% B	
10.00		0% B	
15.00		100% B	A gradient to confirm that the column is clean. A peak of about 0.004 appears to come out. It is likely a refractive index artifact.
15.00		10% B	To get ready for normal use.
17.5		10% B	
End Method			

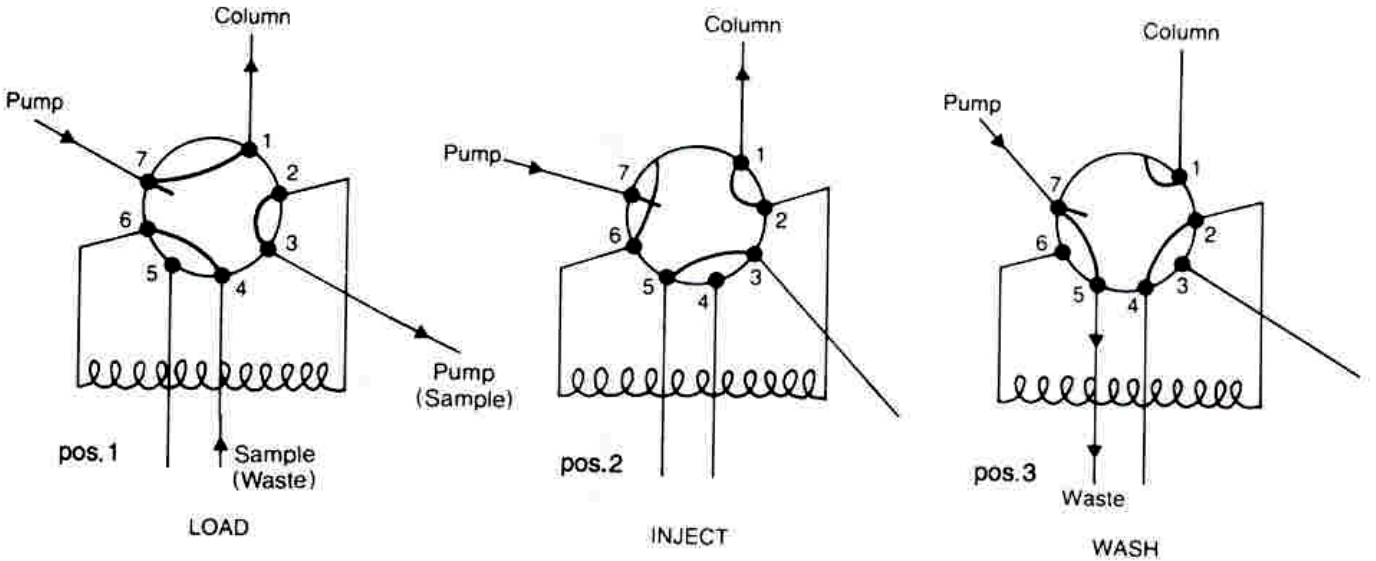
Purification of Oligos (12), Purification Program Larger Equipment
Currently in Bank 2, Method 0.

0.00		10% B	Start with injection valve in Load position
0.00	ml/min	1	Flow rate
0.00	cm/ml	0.5	Chart speed 1 cm = 4 min.
2.00	hold		Insert syringe containing oligo and no air in injection valve, pushing needle through the resistance offered by the rubber membrane and then backing off a little. Open valve in the waste line, and push sample out of syringe, close valve in waste line, remove syringe, change the injection valve to Inject. Press Cont to instruct machine to continue running the program.
4.00		10% B	
4.00	Alarm	0.1	To remind you to set injection valve to Load.
8.00		30% B	A quick ramp up to the relevant region.
8.00	Portset	6.1	Turn on fraction collector.
50.00		80% B	The desired oligo is the last peak off.
54.00		80% B	21-mer elutes at .44% B, 30-mer 50%, 44-mer 55%.

56.00 10% B
End Method

After finishing with one oligo, manually wash column with 100% B.

Manual
100% B, Do Store
0.5 ml/min, Do Store
0.5 cm/ml, Do Store
End when blue pen goes to zero.


Column
Pump
7
1
2
6
3
5
4
Pump
(Sample)
pos. 1
Sample
(Waste)
LOAD
Column
Pump
7
1
2
6
3
5
4
pos.2
INJECT
Column
Pump
7
1
2
6
3
5
4
pos.3
Waste
WASH

Washing Column Before C Prep Larger Equipment

Wash the pumps with C buffer (See card 9 of Purification of Oligos).

Wash the phosphocellulose column (Valves set to number 4)using the procedure described on card 13 of Purification of Oligos and a flow rate of 2 ml/min, using 0%B.

Adjusting Pump Seals on FPLC Larger Equipment

See Sect. 7.1 of the P500 Pump Manual (Folder in the Box).

Set injector valve to Wash so the pump output bypasses the columns. To run the pump manually, press Manual. If you are working on pump A, enter 00, for pump B enter 100 and press Do Store. Then enter 10 and press Do Store. This runs the pump at 10 mls/min. When piston nears the end of the cyliner, press Pause, repeat the above setting the pump rate to 1.0. Note that to start again you must press Cont.

Follow the directions for adjusting the seals.

To test, see Red Handbook, Manual 7, procedure 13, p. 49.

copies of this and relevant pages should be in the folder under pumps.

Have someone show you the first time.

Page through menu, choose self-test and select all (can skip this).

Check that argon pressure in main tank is greater than 500 lbs/in^2, that the low pressure value is about 60 lbs/in^2, and that the waste bottle is not greater than 4/5 full.

Fill the acetonitrile bottle with HPLC grade acetonitrile, following the bottle change routine shown to you or described in the manual. Add other reagent bottles as needed and set the alarms as noted on card 3. Note that the acetonitrile bottle is to be tightened just until it is snug, not overtight, which crushes the washer.

Alarm settings can be adjusted in light of the remaining reagent and the number of cycles used from the bottle since the previous bottle change. The numbers of cycles used is not to be changed except as it is automatically reset to zero when changing a bottle.

Dissolve phosphoramidites in special acetonitrile from the same supplier as the other reagents used, not HPLC grade.

1. Peel back the metal cover of the first phosphoramidite, but don't fully remove it. Stick a black needle through the septum. This will act as a vent.

2. Shift through manual to manual control and turn on Function 2, which flushes argon out the upper column fitting.

3. Use a glass syringe that has been wrapped in aluminum foil, baked and stored in the sterilizing oven. Flush out the syringe with argon three times using the storage connector tube as an adaptor. Finally, fill the syringe with 10 ml argon and attach a black needle without spilling the argon. Inject the 10 ml argon into the bottle of anhydrous acetonitrile, then invert the bottle and withdraw 10 ml of acetonitrile. Inject this into the vented bottle of phosphoramidite.

4. Thoroughly mix the bottle. Examine carefully for undissolved crystals or junk. Either remove all junk or reject such a bottle altogether. Approximately 1 out of 100 bottles must be rejected, and the supplier will willingly take them back. Note that getting junk into the

machine can necessitate a very expensive valve job.

5. When all the phosphoramidites have been dissolved, turn Function 2 off and proceed with the bottle change routine. The following are reasonable starting values for alarm settings.

Reagent	Alarm Settings
#1-5	80
#9	180
#11, 12	340
#14	215
#15	750
#18	78

When finished with a synthesis, remove the column and replace the short adaptor tube in place of the column.

To turn machine off for an extended period of time, see the manual. Use the shut down procedure from the menu, following the prompts and then turn the machine off.

SDS Buffer Protein Gels

SDS buffer	1 x Buffer(1 L)		5 x SDS buffer(1 L)
0.191 M Glycine	Glycine	14.4 g	72 g
0.0247 M Tris base	Tris base	3 g	15 g
0.1% SDS	10% SDS	10 ml	50 ml= 5 gm dry

H_2O to about 800 ml
Adjust to pH 8.3 with HCl
Adjust to 1 liter

20%/0.67% MBA

20% acrylamide	Bio-Rad acrylamide	100 g
0.67% MBA	Methylene-bis-acrylamide	3.35 g

H_2O to 500 ml
Stir until disolved, up to 1 hr
Filter with Whatman paper
Store in refrigerator

Note this is same solution as is used in DNA gels.

30%/0.8%

30% acrylamide	Bio-Rad acryalamide	150 g
0.8% MBA	Methylene-bis-acrylamide	4 g

H_2O to 500 ml

Stir until disolved, up to 1 hr

Filter with Whatman paper

Store in refrigerator

Acrylamide Gels(Protein) Protein Gels

For Hoeffer Apparatus, 0.75 mm thick, makes 5 gels

	Per cent acrylamide, final				
Ingredient	8	10	12	16	14
30% acrylamide/ 0.8% MBA	6	7.5	9	12	10.5
3 M Tris-HCl pH 8.8	2.85	2.85	2.85	2.85	2.85
H_2O	13.35	11.85	10.35	7.35	8.85

10% SDS (electrophoresis grade) 0.22 ml

(If bands are fuzzy bands or polymerization is slow, degas.)

Ammonium persulfate, 100 mg/ml 180 µl (Solutions keep at least a month in the refrigerator.)

TEMED (N,N,N',N'-tetramethylene-ethylenediamine) 11 µl

Pour, gently layer with H_2O.

Polymerizes in .5 to 5 min.

Stacking mix	1 ml
TEMED	1 ml
Am. Persulfate stock	10 ml

Mix in an eppendorf tube and use a 1 ml pipettor to apply.

Stacking mix

Acrylamide/Bis 20%, .67%	6.25 ml
1 M Tris-HCl pH 6.8	6.25 ml
Bromophenol blue	~3 mg
H_2O	37.5 ml

(Bromophenol blue in the stacking gel makes it easy to see the wells and load samples.)

0.123 M Trizma base	0.15 g
4% SDS, electrophoresis grade	0.4 g
1.4 M 2-mercaptoethanol	1.0 ml stock (14 M)
20% Glycerol	2.0 ml
0.2% Bromophenol blue	0.02 g
H_2O	7.0 ml

Store in refrigerator or at -20E in 0.1 ml aliquots

Solution A	Solution B
450 ml methanol	90 ml acetic acid
90 ml acetic acid	450 ml methanol
2.5 gm Coomassie Blue	
450 ml H_2O	450 ml H_2O

Stain 15 min in A (for 0.030", 0.75 mm thickness) and destain in B. Can see bands after 15 min of destaining.

Stain keeps almost indefinitely. When staining or destaining times are prolonged or proteins fail altogether to stain, add some acetic acid and methanol to solution A.

Wash gel 3 x 5 min. in 50 ml water.
Stain with 12 ml Gelcode Blue (Pierce Chem. Co.) 30 min. to 2 hr. Cover with plastic wrap if going more than 45 min.
Destain in 50 ml water.
Bands are barely visible while in stain, more clear after half an hour of destaining, and 2-4 x more intense after overnight soaking in water.

Whole cells: use 200/Cell OD-550 microliters. Spin down, resuspend in 50 microliters, load 15.

Protein: can barely see 0.1 μg in a band of protein with mw > 20,000. At 9, mw, perhaps need 0.5 μg. Optimal, 1 μl of OD_{280}=1, (0.5 μg).

Silver Staining Gels Protein Gels

1. Soak the gel 2 hr to overnight in 50% methanol.
2. Prepare Solution C from Solutions A and B
   A. Dissolve 0.8 g $AgNO_3$ in 4 ml H_20. B. Mix 21 ml 0.36% NaOH with 1.4 ml of 14.8 M NH_4OH (Cold room)
   C. Add A dropwise to B while vigorously stirring, then immediately bring to a final volume of 100 mls and use within 5 min.
3. Gently shake the gel 10 min in C ensuring it doesn=t rest on bottom.
4. Rinse with H_2O and soak 2 min. in H_2O, do twice
5. Mix 2.5 ml of 1% citric acid with 0.25 ml 38% formaldehyde (stock), dilute to 500 ml, add to the rinsed gel, and gently shake for up to 20 min. Stop when almost good.
6. To stop development (takes 30 sec to stop), rinse with ddH_2O, again with 50% methanol and then several times in ddH_2O. If drying down, rinse 4 more times over 24 hrs before drying.

Procedure works well for spotting floor if you're not careful. Use superclean ddH_2O water throughout, $AgNO_3$ need not be super pure.

Vacuum Drying Acrylamide Protein Gels, 0.030", 0.75mm thick Protein Gels

Soak gel 18 hours, not less, with 3-4 changes of H_2O to remove crap

Check that the metal trap and the glass cold finger are not too heavily encrusted with ice. (The ice is removed weekly.) (These traps are in series, first the metal trap, then the glass trap.)

Be sure the mechanical freezer is on and the ethanol in the cold trap is at least -10E. Cold finger from freezer chills the bucket of ethanol in the metal trap in which the glass trap also sits.

Pre-warm dryer (Optional, only speeds the process).

Cut Whatman 3MM paper to fit gel.

Transfer gel to filter paper, place on metal grid, cover with Saran Wrap and place the mylar sheet on top of this.

Dry on Bio-Rad dryer with vacuum on 15 to 20 min (without prewarming).

Warning, do not release vacuum until gel is fully dry. Sandwich order is metal-paper-gel-Saran Wrap-mylar sheet-rubber top.

Stained gels can easily be copied or stained while wet. Place on the glass of the copier or scanner close the lid, but do not allow it to touch the gel. The lid can be conveniently supported a short distance above the glass and above the gels by using two petri plates as spacers. Adjust the darkness to whatever is desired.

Assemble sandwich for each gel using the white alumina plates in back, glass in front. Note the tiny ridges that prevent spacers from sliding between the plates. It is most important that the bottom of the spacer, the glass plate, and the alumina plate all be flush. After inserting in the clamps, check. Overtightening the screws will break a plate. Place one or two sandwiches in the apparatus and use black knobs to fully force the sandwich assembly into the soft rubber base. Insert comb and mark glass 0.5 cm below end of teeth. Remove comb for pouring.

Make gel according to recipies on cards and apply with 1 ml eppendorf or pasteur pipette. Fill to mark and add about 1 ml water, applying to about four evenly spaced positions across the gell. After polymerization, about half an hour, the water can be poured off, and the stacking gell applied. Gels can be wrapped in a water saturated paper towel and Saran wrap and kept for weeks.

Making and Running Gels with Hoefer Apparatus (2) Protein Gels

To run a gel, clamp one or two gels in running apparatus. Add buffer behind sandwich as high as possible and add to bottom reservoir. If only one gel is being run, keep buffer from reaching the electrode wire that would be behind the gel sandwich, if the second one were being used.

For ten wells per gel, samples (Boil 2-5 min. with sample buffer first.) can be up to 10 μl, but 5 is better. They can be loaded with the pipetman.

Electrophorese at 150 V for 1.5 hr for a 12% gel.

Concentrating Protein Samples

Mix sample with an equal volume of 10% TCA.
Vortex and let sit 5 min.
Spin in microcentrifuge 5 min.
Aspirate off supernatant.
Add four drops acetone.
Spin.
Aspirate off.
Add desired volume of sample buffer, heat, and load.

This is useful for drying and preserving coomassie and silver stained gels. Vacuum dried gels frequently craze.

Stain and destain as usual.

Soak in dH_20 2 x 60 min.

Soak 60 min in:

30% ETOH
5% glycerol
1% 2-propanol (isopropanol)

Uses two cellophane sheets a sheet of 3/8" lucite, and a second lucite sheet with a rectangular hole cut in the middle large enough for the gel to be dried.

Cut two cellophane sheets (Obtained from Hoeffer and used for drying gels.) to size and thoroughly wet with water.

Assemble sandwich in the order of: flat plate, cellophane sheet, gel, cellophane sheet, top frame.

Air Drying Protein Gels (1 of 2)

Smooth out any bubbles and clamp lucite sheets together with four black clamps.

Place gel in floor incubator in front of hot air output and dry at 45E for about three hours.

After drying cut excess cellophane, leaving about 1" around gel.

Silver Destain Protein Gels

Useful both for reducing extent of silver staining or removing silver staining altogether, or for removing silver stained spots on the floor.

Solution A: Dissolve 3.7 gm NaCl and 3.7 gm $CuSO_4$ in 85 ml dH_2O. Add concentrated NH_4OH (do in hood) until a deep blue precipitate forms and then dissolves. Adjust volume to 100 ml.

Solution B: Dissolve 4.36 gm sodium thiosulfate in 90 ml dH_2O. Adjust volume to 100 ml.

Procedure: Mix an equal volume of Solution A with Solution B. Dilute one volume of this mixture with three more volumes of dH_2O and use immediately. Soak a silver staind gel for 30 seconds to one minute to clean up background or 2-3 hours to remove all silver staining. Stop by soaking in water.

A destained gel can be restained by soaking in destain 2-3 hours and dH_2O for 30 min. methanol soaking is not necessry.

TAE Buffer for Agarose DNA Gels

TAE buffer	10 x TAE buffer	
40 mM Tris	Tris base	96.0 g
5 mM Na^+-acetate	Na^+-acetate	8.2 g
1 mM EDTA	EDTA, free acid	7.6 g

H_20 to a little less than 2 liters.
Adjust pH to 8 with acetic acid.
Adjust to 2 liters.

TBE Buffer for Acrylamide(DNA)

TBE buffer	10 x TBE buffer	
90 mM Tris	Tris base	109 g
72 mM boric acid	Boric acid	46 g
2.5 mM EDTA	EDTA free acid	7.3 g

H_2O to a little less than 1 liter.
Adjust to pH 8.3 by adding boric acid Tris base.
Adjust to 1 liter.

20%/0.67% MBA

20% acrylamide Bio-Rad acrylamide	100 g
0.67% MBA Methylene-bis-acrylamide	3.35 g
H_2O	to 500 ml

Stir until disolved, up to 1 hr

Filter with Whatman paper

Store in refrigerator

Note, this solution is also used in protein gels.

Acrylamide-MBA Sequencing Gel DNA Gels

38%/2%MBA

38%	acrylamide Bio-Rad acryalamide	190 g
2%	MBA Methylene-bis-acrylamide	10 g
H_2O		to 500 ml

Stir until disolved, up to 1 hr

Do not filter

Store in refrigerator

Acrylamide Gels(DNA) (100 mls) DNA Gels

Ingredient	Per cent acrylamide 3.5	5	6	6 Mini	8	10
20% acrylamide/ 0.67% MBA	17.5 ml	25	30	7.5	40	50
10 x TBE	10 ml	10	10	2.5	10	10
H_2O	72.5 ml	65	60	15	50	40
EthBr 1 mg/ml, optional	100 µl	100 µl	100 µl	25 µl	100 µl	100 µl

Degas 2-10 min.(Optional. If polymerizes slowly or bands are fuzzy, degass.)

Freshly disolved ammonium persulfate, 50 mg/0.5 ml H_2O; 0.4 ml, 0.1 ml Mini.

TEMED (N,N,N',N'-tetramethylene-ethylenediamine) 25 µl, 7 µl Mini.

5 x Concentrated Sample Buffer(DNA)

0.1% bromomophenol blue	Bromophenol blue (Fisher Chem. Co.)	10 mg
0.1% xylene cyanol	Xylene cyanol (Sigma Chem Co.)	10 mg
50% glycerol	Glycerol	5 ml
H_2O		5 ml

Store in refrigerator in 1 ml aliquots

Drying Acrylamide Gels DNA Gels

Check that the metal trap and the glass cold finger are not too heavily encrusted with ice. (The ice is removed weekly.) (These traps are in series, first the metal trap, then the glass trap.)

Be sure the mechanical freezer is on and the ethanol in the cold trap is at least -10E. Cold finger from freezer chills the bucket of ethanol in the metal trap in which the glass trap also sits.

Cut Whatman 3MM paper to fit gel.

Transfer gel to two layers of filter paper, place on metal grid, cover with Saran Wrap and place the mylar sheet on top.

Dry on Bio-Rad dryer with vacuum 30-90 min. Warning, do not release vacuum until gel is fully dry. Sandwich order is metal-paper-gel-Saran Wrap-mylar sheet-rubber top.

Warning, gel can crack severly if vacuum is released before the gel is dry.

Electrophoresis Conditions DNA Gels

	I(ma)	V(volts)	T(hr)
Horizontal Mini			
1%-Agarose	(50)	50*	90 min
6%-Acrylamide-DNA	(50)	100	45 min
10%-Acrylamide-Protein	(50)	60	2 1/2 hr
Horizontal			
1%-Agarose	(100)	100	4
6%-Acrylamide-DNA		150	1 1/2 to 2
Vertical			
-Acrylamide-DNA			
12%-Acrylamide-Protein			
Gel Binding Assay			
full size	(45)	160	1
mini	(20)	50	60 min

Adjust voltage as indicated, current should be near value in parentheses.

Acrylamide Sequencing Gel DNA Gels

For half width gels, use half the amounts.

	Per cent acrylamide		
Ingredient	8%	20%	6% long
Urea	48 g	44 g	80 g
H_2O	37 ml	7 ml	52 ml

Heat in microwave then shake, heat etc. until urea is dissolved.

38% acryl./2% Bis	20 ml	50 ml	24 ml
10 x TBE	10 ml	10 ml	16 ml

Millipore filter (Optional), 47 mm dia. 0.45µ. (Fuzzy bands can result if filtering is omitted.)

Degas, (Optional, but omitting can cause fuzzy bands. Note that filtering simultaneously degasses.)

Amm. persulfate, 100 mg/ml	0.7 ml	0.7 ml	1.38 ml
TEMED	20 µl	20 µl	25 µl

Formamide-bufer-dyes for Sequencing DNA Gels

80% (v/v deionized formamide	800 µl formamide
50 mM Tris-borate pH 8.3	50 µl 1 M Tris-borate pH 8.3
1 mM EDTA	4 µl 0.25 M EDTA
0.1% xylene cyanol blue	50 µl 2% xylene cyanol blue
0.1% bromophenol blue	50 µl 2% bromophenol blue
	50 µl H_2O

Stir formamide with amberlite MB-1 mixed bed resin for 2 hours to deionize, then filter through Whatmann paper.

Clean plates with Ajax powder, then ethanol, and dry with paper towels.

Each time siliconize the lower plate (has attached buffer reservoir) by applying 1-2 mls of Stratagene Acrylase siliconizing solution, spreading evenly with a Kimwipe and wiping while drying.

For a half width gel make 70 mls of acrylamide, and for full width, 100 mls.

Lie the lower plate with side spacers in place face up on the bench.

With one hand slightly lift the bottom end of the lower plate and pour all the acrylamide solution onto the center of the plate. While doing this, adjust the height of the bottom end so that the gel solution does not run off either end of the plate.

Place the bottom of the top plate about one inch from the bottom of the lower plate at an angle of about 45E. Gently lower the top plate onto the bottom plate allowing the solution to fill the volume between the two plates. If air is trapped between the plates, raise the upper plate a bit to work the air out. After the top plate has

been fully lowered, slide the top plate back so the bottoms of the two plates are flush. Insert the comb and clamp the plates together with at least four binder clips.

Use Schleicher & Schuell Membrane NA45, a DEAE membrane stronger than paper. Manufacturer states capacity is about 20 μgm/cm^2, but we have seen 4 x this capacity.

Soak 10 min in 10 mM EDTA pH 7.6

Soak 5 min in 0.5 M NaOH

Wash 4 x in distilled water and store in distilled water at 0E.

Electrophorese, locate band, usually do electrophoresis in presence of ethidium bromide.

Insert NA45 paper just ahead of band. To do this it is convenient to epoxy sections of a razor blade as broad as a gel lane to each leg of a pair of forceps. Place the paper between the blades, insert the "sandwich" in the gel just ahead of the band, partially release the pressure holding the blades together and gently withdraw the blades, leaving the paper, hopefully, behind in the gel. Usually the paper has to be pushed back to the bottom of the slot.

DEAE Isolation of DNA Fragments from Gels (2) DNA Related

Run the DNA into the paper. In an agarose gel which has been run at 50 volts, 100 volts for 10-20 minutes suffices. Check that DNA has bound by wiggling the paper under the UV lamp and see if the DNA wiggles.

Trim off paper without bound DNA.

Do not let paper dry before removing DNA.

LNET

150 mM NaCl	0.9375 ml 4 M NaCl
0.1 mM EDTA	12 µl 0.2 M NaEDTA
20 mM Tris	0.5 ml 1 M Tris-HCl pH 8
	23.6 ml H_2O

DEAE Isolation of DNA Fragments from Gels (3) DNA Related

HNET

1 M NaCl	6.25 ml 4 M NaCl
0.1 mM EDTA	12 µl 0.2 M NaEDTA
20 mM Tris	0.5 ml 1 M Tris-HCl pH 8
	18.25ml H_2O

Rinse paper with bound DNA 2 min in LNET.

Add paper to a minimum volume of HNET to which arginine-HCl has been added to 10 mg/ml and incubate at 65E for 2 hr.

Ethanol precipitate 2 times.

The above works well to elute fragments up to 1 kb. For larger fragments, double the arginine concentration to 20 mg/ml and readjust the pH if necessary or double the Tris as well and incubate 4 hr.

Electroelution Into Dialysis Tubing (1 of 2) DNA Related

Electroelution provides a reliable general method for isolating large or small amounts of DNA out of acrylamide or agarose gels with a recovery >50%.

Take as small a piece of gel as possible containing the DNA.

From a stock of #8 dialysis tubing prepared and stored as described in "Practical Methods", cut a piece about 2.5 inches long.

Close one end with an orange clamp, Spectra/Pore Closure Closure (VWR), currently kept under the spectrophotometer.

Rinse the inside twice with TBE or TAE buffer, depending on which your electrophoresis used.

Insert your gel slice and close the other end as close to the gel slice as possible. Generally you will have less than 0.5 ml of buffer in the sack. Check for leaks by squeezing gently. Redo if it leaks.

Place in electrophoresis tank with buffer slightly above the sack, and electrophorese 30 to 60 min with the electric field perpendicular to the sack at a similar v/cm as the original electrophoresis.

Reverse the current for 30 seconds. Then remove the sack from the tank, and, while wearing gloves, gently kneed the sack to help dislodge any DNA stuck to the walls.

Open one end and clip away excess tubing. Remove buffer with a pipettor after flushing the inside walls of the tubing several times to dislodge any DNA still stuck to the sack.

Ethanol precipitate.

Tips: Remember to label the clamps if you are eluting multiple samples. Never let the tubing dry out during the elution. If you elute from agarose subsequent enzymatic reactions may work better if you phenol extract after elution. If you have the space, flushing the extraction tubing with more fresh buffer slightly increases yields.

Electroelution from Gels(Hoeffer Elution Apparatus) (1 of 2) DNA Related

Store the apparatus in immersed in distilled water. Occasionally replace the dialysis tubing at the bottom of the chambers with boiled number 20 tubing. If if doubt change it, it takes five minutes.

Check that neither chamber leaks by filling each 3/4 full and waiting a few minutes to see if any drops form on the bottom of the chambers.

Place the chamber on the raised central portion of a small gell reservoir apparatus. Place disc of white plastic screen that is part of the apparatus on the ledge near the bottom of the small chamber. Add buffer to both chambers to a height of about 1/4 inch above the central portion of the apparatus. Thus, there is a liquid connection between the chambers. Be sure sufficient buffer is in the gel reservoir to contact the bottoms, (dialysis membranes) of the two chambers and that there is no bubble beneath either membrane.

Add the slice of gel containing the DNA to be eluted to the side with the small chamber and center the slice on the white screen.

Electroelution from Gels(Hoeffer Elution Apparatus) (2) DNA Related

Run the gel current at the same voltage as used in the electrophoresis for about an hour. Carefully remove the buffer from the apparatus until the level is below the central portion. Then remove buffer down to the white screen. If possible, check that most of the DNA has been eluted out of the gel slice.

Place the apparatus in a tray of distilled water for 20 minutes. Use a pipette to flush the buffer inside the small chamber over the inside of the membrane surface and remove from the apparatus.

Ethanol precipitate the DNA.

Geneclean Extraction of DNA (1 of 2) DNA Related

This is good for extraction of ds fragments greater than 500 bp from any type of agarose or out of PCR solutions containing nucleotides, RNA, or other contaminants, i.e. PCR reactions. Recoveries of physical and labelled quantities are usually greater than 50%.

The TBE Modifier, NaI, and glass beads (glass milk) are supplied in the kit and are kept in the storeroom. NaI both helps melt the agarose and participates in the binding and dissociation of the DNA from the silica.

Excise DNA band from gel, weigh and transfer to plastic tube.

If sample is in agarose that was run with TBE buffer, add 1/2 volume of TBE Modifier and 4.5 volumes of NaI, otherwise add 3 volumes of NaI.

Incubate at 55E 5' or until all agarose (if present) is melted.

Resuspend the glass milk by vortexing. This works best if the tube is horizontal, and if the tube has been stored horizontally.

Geneclean Extraction of DNA (2) DNA Related

Add 5 µl glassmilk for <5 µg DNA plus 1 µl per each additional .5 µg.

Incubate on ice 10 min, mixing every 1-2 min.

Spin 5 sec. and transfer NaI supernatant to another tube and save in case of problems. Spin the pellet again and remove all possible NaI supernatant.

Wash pellet 3 x with 350 µl ice cold NEW, each time resuspending pellet, and then spinning 5 sec.

Spin 5 sec, remove all traces of NEW and resuspend pellet in TE, 2 x the glass milk volume originally used.

Spin 30 sec to make a hard pellet and remove supernatant which contains your DNA. Repeat extraction of the glass bead pellet.

NEW buffer is 200 mM NaCl, 20 mM Tris pH 7.8, 2 mM EDTA mixed with an equal volume of ethanol.

Deprotection of Oligos, Cpg Long Chain Alkylamino (lcaa) DNA Related

Put a plastic 1 ml syring on one end of column.

Pull 0.2 to 0.3 ml conc. cold NH_4OH (not over 3 wks old, kept in cold room) in another syringe and put in other end of the column.

At room temperature move the NH_4OH back and forth several times.

Let sit 1-2 minutes, then repeat moving the NH_4OH back and forth.

Repeat the above two steps five times.

Withdraw the NH_4OH in one syringe and put eppendorf tube.

Attach a cap sealer to hold cap closed and incubate at 55E eight hours to overnight or at 80E for 45 min.

Chill on ice and then aliquot 0.1 ml portions into eppendorf tubes.

Add 1 ml n-butanol to each tube, vortex 15 sec, spin in cold room 1 min.

Remove supernatant.

Wash precipitate with 95% ethanol and remove ethanol.

Lyophilize 30 seconds to dry.

CsCl Plasmid Prep DNA Related

Innocule 600 ml of YT (+ antibiotic) with 1 ml ON culture.

Grow 24 hours or grow to OD about 1, about 3 x 10^8/ml and add chloramphenicol to 25 μg/ml and grow for 22 more hours.

Spin down cells in GSA bottles, 7K 10 min, done at 4E.

Resuspend cells in 20 ml 4E TE.

Spin down in the glass walled Sorval tubes, 7K 10 min, 4E.

Resuspend pellet in 2 ml 25% sucrose, 50 mM Tris-HCl pH 8.0. Add 0.4 ml of 10 mg/ml freshly dissolved lysozyme in 0.25 M Tris-HCl pH 8.0.

Mix thoroughly but gently with a glass rod and let sit 10 min on ice.

Add 1 ml 0.25 M EDTA pH 8.0, ice cold, mix, let sit 10 min.

Add 3.2 ml w/v Brij, 0.4% w/v DOC, 0.06 M EDTA, 50 mM Tris-HCl pH 8.0.

Mix, let sit on ice 10 min.

Centrifuge in glass Sorval tubes 18 K 45 min.

Pour supernatant into 3 x 5/8 polyallomer tube with 6.8 g CsCl.

CsCl Plasmid Prep (2) DNA Related

When CsCl is in solution, add 0.4 ml 15 mg/ml EthBr in H_2O.

Adjust refractive index to 1.3925 $\pm$ 0.005 with H_2O (1 ml H_2O added decreases the index by 0.0068.)

Centrifuge 45 K at 20E for 18 hr in angle 65 rotor.

Shine UV light from side and collect the lower fluorescent band.

Invert the column several times to resuspend the gel.
Remove top and bottom caps and allow excess buffer to drain out.
Place column in one of collection tubes.

Centrifuge 2 min in swinging bucket clinical centrifuge or horizontal rotor 8,000g max.

Discard collection tube and buffer collected.
Apply up to 50 µl to center of gel bed.
Place column in fresh collection tube and spin 4 min.
Purified sample is in collection tube.
Note, collection tubes cannot handle phenol/chloroform.
The columns are equilibrated in 10 mM Tris, pH 8.0, 0.1 M NaCl.

Single-strand DNA Prep DNA Related

Inoculate 2-5 ml YT from a colony, add 20 µl of M13 phage stock and grow 12-24 hr. at 37E. (80% reliable) For greater reliability, grow cells until their turbidity can just be seen, then add M13. Colonies to be used must be fresh, a day or so old is OK, more is not, yields will be poor when colonies are old.

After growth spin one ml of culture in microcentrifuge five min.

Add supernatant to microcentrifuge tube containing 400 µl of ice cold 2.5 M NaCl 20% PEG 8000.

Vortex and leave on ice more than 30 min.

Spin 10 min in cold room, discard sup.

A tiny pellet should be visible here, if not start over.

Dissolve pellet in 200 µl TE.

Phenol extract, $CHCl_3$ extract, spinning five min.

Ethanol precipitate with 2 volumes ethanol and .1 volume 3 M NaOAc.

Wash pellet with 95% ethanol, dry, and resuspend in 9 µl TE.

Run 2 µl on .7% to 1% agarose gel and use rest for sequencing.

Inoculate 2-5 ml medium with a small amount of F= cells and 20 µl M13 stock.

Grow at 37E 20-24 hours.

Spin down 1 ml cells 5 min in eppendorf tube.

Heat supernatant to 65E for 15 min.

Store at -20E.

From a freshly transformed colony, inoculate 250 ml of YT plus antibiotic in a 1 liter flask at 3 PM. Since chloramphenicol is not used, timing is important since cells must be at stationary to build up plasmid. If colonies are old, they don't start up in time. Next morning harvest cells, GSA rotor, 5K, 10 min, 4E. Can use one bottle.

Pour off sup, resuspend really well in 6 ml 0E TGE, (Miniprep solution 1)

25 mM Tris-HCl, pH 8.0
50 mM Glucose
10 mM Na-EDTA, neutralized to pH 7.0 to 8.0.

Transfer to 50 ml Sorval polypropylene tube.

Add lysozyme to about 2 mg/ml and incubate on ice water 20 min.

Add 12 ml freshly made NaOH-SDS, (Miniprep solution 2)

0.2 M NaOH
1% SDS.

Mix gently by turning upside down twice, and incubate 10 min on ice water.

Add 7.5 ml 0E acetate solution (Maniatis, Miniprep solution 3) to precipitate SDS as K-SDS.

60 ml 5 M KAc
11.5 ml glacial acetic acid
28.5 ml H_2O.

Incubate 20 min on ice water.

Spin 10 K, SS34 rotor, 20-30 min, 4E. The pellet may be loose, so pour the supernatant (containing plasmid) through gauze into a clean centrifuge tube.

Add 100 μg RNaseA. This is DNase free RNase A prepared by boiling as described by Maniatis. Make a 10 mg/ml stock and use for years.

Incubate at 37E for 30 min.

Extract once with phenol-chloroform at room temp. and spin 5 K, 5 min, SS34, 20E.

Split supernatant into two tubes.

Medium Scale PEG Plasmid Prep(3) DNA Related

Ethanol precipitate by adding 2 volumes of 95% ethanol, no salt. Shake vigorously and let sit 2 min at room temp.

Spin 12K, 15 min, SS34, decant supernatant, rinse briefly with 95% ethanol to dry pellet, dry pellet in lyophilizer 5 min.

Resuspend pellet in 1 ml TE, can heat to 65E to help resuspend.

Transfer to 1.5 ml eppendorf tube. Add 500 µl 20% PEG 8000 in 2.5 M NaCl.

20 gm PEG
14.5 gm NaCl

Bring to 100 mls and spin out any junk. Store in refrig.

Shake and vortex, and incubate 20 min at 0E.

Spin in microfuge 10 min, 0E.

Suck off all supernatant with drawn out pasteur pipette connected to aspirator. Can do at lab bench as pellet is firm.

Resuspend each pellet in 500 µl TE. This is hard to do. Usually need to incubate at 65E 15 min. and vortex a lot. Proteins which are carried over don't resuspend.

While still hot, add 500 µl room temp. phenol-$CHCl_3$, vortex, spin 2 min. at 20E.

Repeat phenol extraction until no more protein is present.

Ethanol precipitate, room temp. Add 3 M NaAc (pH at 6.0 to enhance precipitation) to 0.3 M, and 1 ml ethanol. Spin 10 min. room temp, dry.

Resuspend each pellet in 150-200 µl TE,

Total procedure takes 4-5 hours.

pBR plasmids yield 200-400 µg, pUC plasmids yield 400-750 µg.

On an overexposed 1% agarose gel, should not see any RNA. If it isn't free of RNA, go back to the RNase step and work to the end.

Add 11.5 µl dd H_2O
2 µl 10 x kinase buffer
1 µl of oligo at 200 ng/µl
5 µl γ-ATP (NEN, 6000 Ci/mmol, 10 mCi/ml)
1 µl T4 polynucleotide kinase (20-40 u/µl.

Incubate 45 min at 37E.

Precipitate by adding
67 µl 0.3 M Na-acetate
267 µl ethanol.

Incubate 1 min, then spin 15 min. at room temp. in the microcentrifuge in the radioactivity area.

Remove supernatant with a P1000. Pellet should be .2K on the 100 x scale of the geiger counter, and the supernatant .1K.

For some applications, can reprecipitate by adding 250 µl 0.3 M Na-acetate pH 6.0 and 750 µl ethanol and spinning.

Wash pellet, 95% ethanol, dry in lyophilizer and resuspend in TE or H_2O.

Wear gloves, work behind shield, put sup. in beaker in lead shield, put pipette in container in plastic shield.

1-5 μg of DNA with 2 fragments should have counts in pellet of more than 3K on the 100 x scale. Throughout these operations wear badge, monitor everything for radioactive spill.

CIP Treatment of DNA

Good for up to 10 μg vector.

Add 2-3 u CIP to digested vector. CIP is active in most restriction enzyme buffers, including universal restriction buffer.

Incubate at 37E at least 30 min.

Remove CIP by gel purification or phenol extraction. For phenol extraction, add an equal volume of phenol, vortex, bring to 65E in waterbath, vortexing a few times over five minutes. Be careful to have top tight as spilled phenol is no fun. Spin, take top phase and do a normal phenol extraction.

An ether extraction is sometimes useful before ethanol precipitation.

10X CIP Buffer

0.5 M Tris-HCl pH 9.0	0.5 ml 1 M Tris-HCl pH 9.0
10 mM $MgCl_2$	10 μl M $MgCl_2$
1 mM $ZnCl_2$	1 μl 1 M $ZnCl_2$
10 mM Spermidine	10 μl 0.1 M Spermidine
	0.5 ml dH_2O

5 X Taq Sequencing Stock

250 mM Tris-HCl pH 9	250 µl 1 M
50 mM $MgCl_2$	50 µl 1 M
100 µM dGTP	1 µl 100 mM (Pharmacia stock)
50 µM dCTP	0.5 µl 100 mM
50 µM dTTP	0.5 µl 100 mM
50 µM dATP	0.5 µl 100 mM
dH_2O	697.5 µl

2X ddNTP Mixes

ddNTP:	A	C	G	T
5X Taq seq. stock	40	40	40	40
ddNTP (Pharmacia, 5 mM stock)	12	8	2	12
dH_2O	48	52	58	44

PCR Sequencing (2) DNA Related

Sequencing Stock

8 µl template DNA (Wizard Miniprep, 50 µl)

5 µl primer, approx. 4 ng/µl, kinased (200 ng/50 µl, giving 20 x 10^6 Cherenkov counts per min)

0.4 µl Taq (5u/µl)

8 µl dH_20

Sequencing Reaction

5 µl sequencing stock

5 µl 2X ddNTP mix

Overlay with mineral oil

Cycle in PCR machine, 29 cycles 94E 1 min, 60E 1 min, 65E 1 min

5 µl Formamide-buffer-dyes

Heat to 95E 5 min and chill on ice before loading 7.5 µl.

Provides readable sequence from about 40 nucleotides beyond primer to about 200. Can see bands after one hr on phosphorimager, but expose O.N. for film.

If the film is completely clear, look for the tiny AKodak Safety Film@ printing at the edge. If the film or developer are bad, this will be absent.

Premature termination in PCR sequencing can be caused by the presence of K+ ions, particularly if contiguous G=s are present. See NAR 23, 539 (1995). Skip monovalent cations and use 80 mM Tris-HCl pH 9.3 and $MgCl_2$ at about 4 mM.

PCR Colony Screening DNA Related

Place 27 µl PCR buffer in a small PCR tube.

Add, or have present, 50 ng each of the amplifier oligos.

With a P-20 pipettor, poke a colony and disperse in the PCR buffer the 2-5 µl of cells that stick and goes in the hole in the tip.

Add a drop of mineral oil.

Lyse cells by heating to 100E 10', (currently file 58 is 100E 10' and cools to 22E in 10', also TLH1 on Mini 1).

Add 0.25 µl Taq enzyme, being careful to inject it under the oil. For small numbers of samples, a Hamilton microsyringe with a plastic tip made from PEI tubing is useful. For larger numbers, dilute Taq in PCR buffer and use a P-20 to add 1-5 µl of the diluted enzyme.

Do a normal PCR run of 25 cycles, 95E-60E-72E.

Run 15 µl on a 6% gel when looking for typical 100-1000 bp fragments.

Be sure to include the original plasmid as a control.

Sequencing Downtown (1 of 2) DNA Related

Grow overnight culture in 3-5 mls YT medium plus antibiotic.

Do Promega Wizard Miniprep, eluting in a final volume of 35 µl dH_2O.

Read OD at 260 and 280 nm. DNA is good of OD 260/280 > 1.2. Use conversion of 50 µg/OD_{260}. DNA concentrations range from 100 to 1000 µg/ml

For plasmids on the order of 5 kb, add 2400 ng plasmid DNA and 1 µl of 12 µM primer in total volume of 24 ml, make up with dH_2O, in 1.5 ml eppendorf tube. Label top of tube, e.g. RFS2-54A.

Samples for DAF sequencing facility are picked up from Corces refrigerator at noon on Mondays and Wednesdays. Samples can also be hand delivered to DAF.

Sent samples must also be entered into system via the net,
http://finch.daf.jhmi.edu/Finch
Login: schleif_student and password 2005arac for undergraduates
schleif_roton and password 2005arac for rotators.
On left, click "Sequencing, New Request" and fill in request

information, DNA type being plasmid. Pick our id number from the Bill to pull down menu and then click on tube request.

Identify samples on tube request. Select a tube and enter primer and template, which should be the same as the tube label used above, e.g. template is RFS2 and primter is 54A. Then click on set selected, which should result in a DNA molecule appearing in tube.

When all tubes that were sent have been identified, click on view all to check for errors and then click on add new request.

Log off with door symbol in upper right.

Grow overnight culture in 3-5 mls YT medium plus antibiotic.

Do Promega Wizard Miniprep, eluting in a final volume of 30 µl dH_2O.

Read OD at 260 and 280 nm. DNA is good of OD 260/280 > 1.2. Use conversion of 50 µg/OD_{260}. DNA concentrations range from 100 to 1000 µg/ml. Typically read 5 µl of sample in 1 ml cuvette. When in doubt, send sample for sequencing. Usually it works even when OD suggest there may be a problem.

For plasmids on the order of 5 kb, add 2000 to 3000 ng plasmid DNA and 4 µl of 12 µM primer in total volume of 20 µl, make up volume with dH_2O, in 1.5 ml eppendorf tube. Label top of tube, e.g. RFS2-54A.

Samples are picked up daily at 4 PM from office. Put them in a baggie from the container on the left above the office fax machine. Staple the project description form to the baggie and put in the FedEx envelop on top.

Project Description forms, (Obtain partially completed blank from Lab Info folder on the office computer.) Put the name Mike, Schleif, or

SeqWright Sequencing (2)

Rotator in the customer information block and fill in your own email address and check the proper template block.

Make a copy of the project description form and put it in the orders tray so we can prove to the bookkeepers that we actually had the sequencing done. Also enter the transaction in the orders database on the computer.

You will be emailed the web site from which you can pick up your sequence information.

Web site: https://www.seqwright.com/ssl/RobertSchleif/Mike
/Rotator
/Schleif

User name: Rschleif
Password: nyquist

Hot DNA for Gel Binding Assay (1 of 2) DNA Related

Label 200 ng of Aforward@ oligo (See Oligo labeling card). Resuspend final product in 50 µl dH_20.

PCR amplify in 100 µl total volume.

62 µl H_20
10 µl 10 X PCR buffer
1 µl 10 ng/µl template DNA
25 µl labelled oligo from above
1 µl reverse oligo at 100 ng/µl
1 µl Taq polymerase
Oil

Typical cycle parameters are:

93E 1 min.
45E 1 min.
72E 1 min.
30 cycles
72E 10 min. and then hold at 4E (Martin in Mini 1 is good.)

Remove buffer without oil to a fresh eppendorf tube.
Extract with an equal volume of phenol/chloroform.
Ethanol precipitate, resuspend in 50 µl TE.
Measure the radioactivity in 1 µl by Cherenkov radiation in scintillation counter (Group 4).
Use 10,000 counts per binding reaction.

Some folks prefer a two-fold excess of cold primer above hot to avoid presence of any hot single-stranded DNA.

Quick Change Mutagenesis (1 of 2) DNA Related

This is a good method for making site-directed point mutations.

Insertions of 12 aa and deletions of 40 aa have been made easily.

Primers

Both of the mutagenic primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid.

Provide at 12-18 correct bases on either side of the mutation and begin and end with G or C.

Reaction

5 µl 10 X PCR reaction buffer (See Stratagene data sheet)

1 µl parent plasmid containing 5-20 ng (0.2 µl standard prep)

1 µl (125 ng/µl) primer 1

1 µl (125 ng/µl) primer 2

1 µl nucleotide mix containing 10 mM each nucleotide

41 µl H_2O

Mix, add 1 µl *Pfu* DNA polymerase 2.5 u/µl mix again,

Overlay with a drop of mineral oil.

Half a drop mineral oil in well of thermocycler. Tube should fit snugly.

Cycle: 95E 1 min., then 18-20 cycles of 95E 30 sec.-55E 1 min.-68E 2 min/Kb of plasmid length, then 4E, QUICKCHAN (Small tube thermocycler) May need to raise 55 to 58 if primers are particularly GC rich.

After the cycling, add 1 μl ***DpnI*** through the mineral oil, mix by pipetting up and down, spin 1 min, and incubate at 37E 2 hr.

Minitransform using 1 μl, standard competent cells, often DH5α

Some plasmids are hard to mutate. Ethanol precipitate the entire reaction product, redisolve in 10 μl H_2O, and use this for transformation.

To troubleshoot, run the raction product on an agarose gel to check for the presence of a clear band the size of your parental plasmid.

MacConkey Ara (Gal) Amp Plates Media and Reference

MacConkey Agar Base 20 gm
H_2O 500 ml
L-arabinose or D-galactose 5 gm

Autoclave
Add 25 mg ampicillin to 5 ml YT tube, vortex, and add to flask before pouring.

MacConkey Ara (Gal) Amp Plates Media and Reference

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H_2O 500 ml
L-arabinose or D-galactose 5 gm

Autoclave
Add 25 mg ampicillin to 5 ml YT tube, vortex, and add to flask before pouring.

X-gal can be used with minimal or rich plates at concentrations up to 40 μg/ml (depending on the levels of β-galactosidase that need to be detected or discriminated for or against). A stock of X-gal at 20 mg/ml in N,N-dimethyl formamide can be kept in the refrigerator

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H_2O 500 ml
L-arabinose or D-galactose 5 gm

Autoclave
Add 25 mg ampicillin to 5 ml YT tube, vortex, and add to flask before pouring.

Stock Reagent Concentrations

Media and Reference

HCl	11.6 M	(36%)
HNO_3	16.4 M	
H_2SO_4	17.8 M	
H_3PO_4	14.7 M	(ortho 85%)
Acetic	17.4 M	(glacal)
NH_4OH	14.8 M	

0.25 M Na-EDTA, pH 8.0

Disodium salt of EDTA (MW 336.2) 8.4 gm/100 ml

Adjust pH to 8.0 with concentrated NaOH while stirring. As you do this, the salt dissolves.

Adjust volume to 100 ml with H_2O

0.2 M K-EDTA pH 7.0

EDTA free acid (MW 292.2) 5.84 gm

H_2O 70 ml

Adjust to pH 7.0 with 5 M KOH (14 gm solid + 50 ml H_2O)

Adjust to 100 ml with H_2O

1% Brij, 0.4% DOC, 0.06 M EDTA, 50 mM Tris pH 8.0 (for plasmid preps)

Brij 35	1 gm
DOC	0.4 gm
EDTA 0.25 M, pH 8.0	24 ml
1 M Tris-HCl, pH 8.0	5 ml

Adjust volume to 100 mls with ddH_2O

TE

1 M Tris-HCl pH 8.0	1 ml
0.25 M Na-EDTA pH 8.0	0.4 ml
H_2O	98.6 ml

Autoclave

20 X SSC

NaCl	175 gm
Na-citrate	88 gm
H_2O	1000 ml

Conc. HCl to pH 7.0, requires approx 1 ml

50 mM $CaCl_2$

$CaCl_2$ (dihydrate) MW 147 0.735 gm/100 ml

Glycerol Vials for Storing Strains

Make 50% glycerol in M10
Add 2.5 ml to 1 dram screwtop vials
Autoclave
Grow cells to early log phase, add 0.5 ml to vial and store at -20

Don't expect cells to survive more than a year by this method.

Carboys(1.33 YT) Media and Reference

Yeast extract	100 gm	(150 mls in beaker)
NaCl	100 gm	(70 mls)
Bactotryptone	160 gm	(225 mls)
H_2O	15 l	
Antifoam	3.5 ml	

Autoclave 1 hr
Allow two hours to cool, i.e. a total of 3 hours in autoclave.
Use extreme caution when removing from autoclave. Burns with this amount of liquid often are lethal.

Min Ara-Fucose Plates

Minimal plates mix	2.95 gm in 125 ml H_2O
Agar	3.75 gm in 125 ml H_2O

Autoclave each of the above separately, be sure to stir up after autoclaving, then add

B_1, 1 mg/ml	2.5 ml
20% L-arabinose	1.25 ml
20% D-fucose	2.5 ml.

Makes about 10 plates

Add any required amino acids.

Innoculate 50 ml YT with 50 µl cells from overnight tube.
Grow to OD_{550} of 0.75 to 0.90, approximately 3 hr.
Spin down cells in cold sterile plastic Sorval tubes, 10 min at 10 K.
Resuspend pellet in 20 ml cold sterile 50 mM $CaCl_2$.
Combine pellets into one tube and incubate on ice 15 min.
Spin 10 min at 10 K.
Resuspend in 5 ml 50 mM $CaCl_2$, 15% glycerol.
Immediately freeze cells in 0.2 ml aliguots in eppendorf tubes in dry ice and store at -70E.

Transformation Media and Reference

Thaw competent cells on ice.
Add DNA to 0.1 ml (10 mM Tris-HCl pH 8.0, 10 mM $CaCl_2$, 10 mM $MgCl_2$) in 13 x 100 mm sterile glass tubes.
Add 100 µl cells and incubate on ice 30 min.
Incubate at 37E 2 min.
Incubate at room temp 10 min.
Add 0.5 ml YT
Incubate 30 min at 37E with vigorous shaking. Put in rack in floor incubator, but incline the tubes one slot, and apply tape to reduce noise.
Spread 0.2 ml on plates or pour 0.5 ml with top agar.

Thaw competent cells on ice.
Add DNA to 50 µl 10 mM Tris-HCl pH 8.0, 10 mM $CaCl_2$, 10 mM $MgCl_2$) in eppendorf tubes.
Add 40 µl cells.
Incubate 25 min on ice.
Incubate 2 min at 37E.
Incubate 10 min at room temperature.
Add 200 µl YT and put at 37E 30 min, (no shaking is required).
Spread 100 µl on selective plates.

You get about 10^6 colonies per µgm plasmid

Phosphate Buffer for β-galactosidase Assay Media and Reference

Dissolve 8.28 gm NaH_2PO_4-H_2O, monobasic (MW 138) in 200 ml H_2O
Dissolve 8.52 gm Na_2HPO_4, dibasic (MW 142) in 200 ml H_2O
Each of the above is a 0.3 M solution

Mix the two together to make a solution at pH 7.0. This requires about 75 ml of monobasic per 200 mls of dibasic.
Freeze in appropriate aliquots at -20E as phosphate buffers rapidly acquire bacterial growth contamination.

Dyes for DNA Gels

0.1% bromophenol blue
0.1% xylene cyanol blue
50% glycerol
H_2O

0.05 ml 2% bromophenol blue
0.05 ml 2% xylene cyanol blue
0.5 ml glycerol
0.4 ml H_2O

Transformation Buffer

10 mM Tris pH 8.0	1 ml 1 M Tris-Hcl pH 8.0
10 mM $CaCl_2$	1 ml 1 M $CaCl_2$
10 mM $MgCl_2$	1 ml 1 M $MgCl_2$
	97 ml H_2O

Universal Restriction Enzyme Buffer

Media and Reference

1 x Buffer	10 x Buffer	
33 mM Tris-Ac pH 7.6	0.33 M Tris-Ac pH 7.6	0.33 ml 1 M
66 mM KAc	0.66 M KAc	0.132 ml 5 M
10 mM MgAc	0.1 M MgAc	0.1 ml 1 M
10 mM DTT	0.1 M DTT	15.4 mg
0.02% Triton X-100	0.2% Triton X-100	2 µl
		0.4 ml H_2O

Grow a 1/1000 dilution of cells in 600 ml YT medium, 2 l flask, 37E, to OD_{600} 0.5 to 0.7.

During the growth it is best to plot OD on semilog paper, 2 cycles.

Chill flask on ice water 15-30 min.

Spin down in sterile bottles, chilled GSA rotor, 5,000 rpm, 10'.

Remove as much supernatant as possible.

Resuspend cells in 600 ml sterile cold water and centrifuge again.

Resuspend in 300 ml sterile cold water and centrifuge again.

Resuspend in 10 ml cold 10% glycerol and centrifuge in SS34 rotor, 10,000 rpm, 5 min, use either cold plastic tubes or heavy wall Sorval tubes.

Resuspend in 1.5 ml cold 10% glycerol.

Freeze in 200 µl aliquots.

Electroporation, Procedure Media and Reference

Thaw cells at room temp and place on ice.

Mix 40 µl of cells with 1-2 µl DNA in cold eppendorf tube on ice and keep there 1-3 min. This can be DNA straight from ligation mix.

Transfer to a 0.2 cm electroporation cuvette that has been in ice at least three minutes.

Smack cuvelle smartly on the bench a few times to be sure the cells are across the bottom of cuvette between the electrodes.

Pulse once (See next card) at 2.5 KV, 25 µF, 200 ohm resistance.

Immediately, within 10 sec, add 1 ml of room temp YT medium with 1000 µl eppendorf pipettor. Draw back into the pipettor the 500-600 µl that you can suck up again and vigorously expel so as to mix the cells and medium thoroughly.

Transfer about 500 µl cells to an eppendorf tube and incubate 60 min. at 37E. No shaking is necessary, just use a waterbath.

Spread on selective plates, 0.1 to 0.3 ml is good.

Expect 10,000 to 1,000 colonies per 10 ng intact plasmid.

Use the HP supply. Be sure it is off before proceeding!
Set power supply to 0-1999 volts.
With normal electrophoresis leads, connect red to red, black to black from the power supply to the electroporator box.
Set electroporator to "Discharge", number 2, turn power supply on.
Place electroporation cell in BioRad cell holder, turn electroporator to "Charge", number 1, for 20 sec, then switch to "Discharge". Charge charges a capacitor, and discharge discharges it through the cell. Listen for the clicks from the power supply that indicate that current is being momentarily drawn.
Another cuvette can be put in the holder and the charge discharge cycle repeated.
Wash cuvettes with water, rinse with ethanol, and dry one minute in lyophilizer, then put cap on and store until next use.

Lysate C Prep (1 of 3) Media and Reference

For cells containing the p13 and p15 (old fashioned) C overproduction plasmid. Grow cells to stationary phase, O.N., in YT amp.

For cells containing the pSE380 overproduction plasmid, inoculate 5 mls YT plus ampicillin with 16 µl overnight cells, grow 1 1/2 hours and add IPTG to 5 mM and grow an additional 1 1/2 hour.

For cells containing the pGBO series of plasmids, do like pSE380, but omit the IPTG.

Spin down cells, 30 sec. eppendorf centrifuge, 1 ml p13 or 5 mls pGBO or pSE380, and resuspend in eppendorf tube in 0.5 ml lysate buffer containing PFSF.

Sonicate 5 X 5 sec. pulses with power setting of 80, with the tubes in ice water. Do not overheat or vortex. Tubes should become only slightly warm at most. You should be able to see some yellowishness of the supernatant after the spin.

Spin 10 min, 4E.

Remove supernatant and add 175 µl glycerol.

Gently vortex with inverted tube.
Freeze in dry ice-ethanol and store at -70E.
Is useable for at least 1 week.
Typical amounts to use in an assay are 1-5 µl.
Beware that host DNA in the extract slows the binding of AraC to the DNA you add in an assay and that the slowing can be acute if arabinose is present.

Lysate C Prep (3) Media and Reference

Lysate buffer

0.1 M Potassium Phosphate, pH 7.4	1 ml 1 M
0.05 M KCl	500 µl 1 M KCl
1 mM EDTA	50 µl 0.2 M EDTA, pH 8
10% glycerol	5 ml 20% glycerol
1 mM DTE	20 µl 0.5 M or 1.5 mg
0.1 mM M $ZnCl_2$	1 ml 1 mM $ZnCl_2$
50 mM L-arabinose	0.375 ml 20% L-ara
H_2O	2.05 ml dH_2O

Dissolve 3.5 mg PMSF in 1 ml isopropanol immediately before use. This solution is 20 X concentrated. Add 25 µl to 0.5 ml lysate buffer at the sonicator.

10 x TBE, 4 Liters

380 g Trizma base
184 gm Boric acid
29.2 gm EDTA
H_2O to 4 liters
Adjust pH to 8.3 with Trizma base

10X PCR Buffer

500 mM KCl
200 mM Tris-HCl pH 8.3
15 mM $MgCl_2$
0.1% Gelatin

2 mM each dNTP

250 µl 2M KCl
200 µl 1 M Tris-HCl pH 8.3
15 µl 1 M $MgCl_2$
10 µl 10%. Melt in microwave first, Sigma G2500
20 µl each, 100 mM stocks pH 7.5 (Sigma XTP=s)
445 µl dd H_2O

Mutagenesis Using XL1-Red Mutator Cells Media and Reference

Thaw LL1-Red competent cells on ice, 100 µl/sample, pre-chill 15 ml Falcon polypropylene tubes on ice, one per sample.

Aliquot 100 µl gently mixed cells into the pre-chilled Falcon tubes and add 1.7 µl β-mercaptoethanol provided with the kit or a fresh 1:10 dilution of β-mercaptoethanol stock in water. Mix thoroughly and incubate 10 min. on ice swirling every two min.

Add 30-50 ng of DNA to the tubes, swirl gently and incubate on ice 30 min.

Put tubes in 42E water bath for 45 sec, and move to ice bath for 2 min.

Add 1 ml 42E YT and incubate at 37E 1 hr, mixing occasionally.

Plate 200 µl of the transformed cells on YT plate with appropriate antibiotic and incubate at 37E 24-30 hours.

Use a sterile applicator stick to innoculate 5 ml YT plus antibiotic with at least 200 colonies (all sizes) from the transformation plates.

Grow overnight and do a miniprep (Wizard) on 1.5 to 2 ml.

Transform the isolated, mutated plasmid DNA into the strain of your choice (CaCl2 method is fine), and screen transformants.

Soak dry G25 overnight in 10 mM Tris pH 8 100 mM NaCl. Make sure to have a great excess of buffer as the volume increase of Sephadex is large.

Pour off supernatant, add more Tris/Nacl and add NaN_3 to 2 nM and autoclave.

Remove plunger and needle from 1 ml syringes.

Pack a bit of siliconized glasswool into the syringe and tamp down lightly with the plunger minus the rubber tip.

Pipete a slurry of the G-25 into the syringes and let excess fluid drain out. Keep adding slurry until it won=t drain, then centrifuge at 2,000 rpm in the swinging bucket centrifuge for 1 min.

Add more slurry and spin and repeat until the packed slurry reaches the 1 ml mark on the syringe.

Top up with Tris/NaCl/NaN_3 and store at 4E.

For use, wash 4 x with desired buffer, often T# + 50 mM KCl. For last spin, go 4 min.

Add sample, 100 µl.

Transfer column to 13 x 100 mm test tube with a 600 µl eppendorf tube in the bottom.

Spin 2,000 rmp for 5 min. Sample will be in eppendorf tube.

Tetrazolium Plates Media and Reference

Cells catabolizing carbohydrate release acid which denatures an extracellular reductase. Thus the resulting colonies are white. Colonies that do not utilize the carbohydrate reduce tetrazolium to a red compound and appear deep red. In practice, carbohydrate plus colonies turn pink over several days whereas negative colonies turn bright red in about 18 hours. Intermediate levels of carbohydrate utilization produce Afish eyes@. About 20% of full expression of the arabinose operon gives red colonies. The color is stable upon prolonged storage. Excessive crowding inhibits the color development. Trust only the colors on plates with less than 500-1000 colonies or of well isolated colonies.

Dissolve 50 mg 2,3,5-triphenyltetrazolium chloride in 1 liter good water.
Add 25.5 g Bacto antibiotic medium 2 (Difco).
Autoclave and cool somewhat.
Add 50 ml 20% sugar and pour plates.

Hints for Visual Molecular Dynamics Molecular Display Program
Import the coordinate file using File, New Molecule. To show the backbone as a red tube: Graphics, Representations, Drawing Method—Tube, Coloring Method-Color ID, No. 1.
To show residues 10, 20, and 30 as green VDW spheres for each atom, in Graphics Representations, click the bottom button Create Rep. Change Drawing Style to VDW. In the Selection Box, type resid 10 20 30, and adjust color as above. Click the Apply button.

To make VMD read the pdb file 1.pdb upon pressing the p key (when the cursor is in the graphics window and the graphics display window is selected). This reading replaces the former coordinates with whatever coordinate values are in 1.pdb but leaves the display orientation, color and whatever else has been chosen unaltered.
In the text window issue the following command
user add key p {animate read pdb 1.pdb}

With VMD generate a series of .bmp files using a script. These files can then be used to generate a movie using Adobe Premier.

The text window of VMS is like DOS and you can issue DOS commands like dir for a directory listing and cd for change directory, i.e. cd .. to go up one directory level, and cd ara to change to the ara directory (possible if ara can be seen from the prompt by issuing a dir command).

A script for VMD should be a text file. It is run by changing the directory of the prompt in the VMD text window to the directory containing the script and then issuing the command Aplay (script name)@.

Note, the text interpreter of VMS is very fussy about bracket location. The following works, but moving a bracket to another line will not.

```
set frame 0
```

```
for {set i 0} {$i < 360} {incr i 3} {
     set filename name.[format "%04d" $frame].bmp
     render snapshot $filename
     incr frame
     rotate x by 3
}
```

To generate avi video using Adobe Premier, open Premier and start new project, select NTSC 720 x 480 Video for Windows, and you get five windows, including Project, Timeline, Monitor, Effect, and Navigator. Import your stills, File > Import > File, select the first of your stills (must be consecutively numbered, file.0001, file.0002 etc., click on numbered stills box and open. Clip is put in Project. Drag to Timeline, use Clip > Duration or Clip > Speed to adjust its duration. Check in Monitor, Export Timeline > Movie.

In Microsoft Powerpoint under File, Page Setup choose a page size with a ratio of 4 x 3. Onscreen presentation, 10 x 7.5 or custom, and adjust to 8 x 6.

In Adobe Illustrator make rectangles with your background color the same dimension as the page size in Powerpoint and have nothing outside the rectangle. For later use, save as usual, and for use in Powerpoint, export in jpeg format. Insert a figure on a completely blank slide using Insert, Picture, From File. If any white space shows around your rectangle, you likely had a drawing element outside the rectangle.

To use bitmapped images, e.g. a screen snapshot of AraC from a graphics program like VMD, first adjust the graphics window displaying your image to have a width to height ratio as close to 4 x3 as you can. Save, (VMD uses rgb format) and insert into Powerpoint as above. Use the handles at the corners to adjust the image size to fill the slide.

To show a avi movie (prepared by Adobe Premier) Insert, Movies and Sounds, Movie from File. Depending on your settings, a popup box may ask

you, or you may have to right click on the resulting slide and click on Edit Movie Object to adjust it to loop continuously or play once only. Similarly, use Action Settings to set the movie to play automatically when the slide is shown (generally the best option) or to play when you mouse over the image or click on a button you place on the image.

Oligo is awkward to use, sorry. It is designed to help choose oligos for PCR amplification when the sequence of the region to be amplified is known. Basically, you can slide a window of 21 (default) or other size across the sequence and pick points that you would like to use for your primer sequence. The program begins with this window at the 5' end of the sequence. The current position of the oligo window is called the current oligo, CO, (The abbreviation is used quite a bit in the program.) The melting temperature and other properties of the current oligo are shown in a box on the right of the screen. A second oligo can also be chosen, and the oligos checked for hairpins, dimer formation, and extraneous binding sites in the sequence.

Use of the program to evaluate a pair of oligos chosen by eye.

On the PowerMac, shift to DOS (and back to Mac) by simultaneously pressing the Apple Key-Space bar, and Return. Change to the Oligo directory

by typing CD oligo at the C> prompt.

At the DOS prompt type oligo.

Press Enter

At this point you must get a DNA sequence into the program. By pressing enter and following the instructions, you can work your way around the hard disk or floppy and choose a sequence to be loaded into the program (The program looks for files of the name *.seq. If it finds any, it lists only these. If it finds files with the name *.DNA or *.RNA, it lists only these. Failing these, it lists all files present.) You can also enter a short sequence by hand if instead of enter, you type .

After entering the sequence, you are presented a flow chart that can be safely ignored, and a box for the length of the oligo you wish to analyze. If you know how long the homology region of your oligo

will be, enter this number. Now you see a crude graph, an oligo and its complement, and a box at the right containing a lot of information about the oligo. The main menu for the program is now entered by typing I (Info).

Enter I

Enter L

Enter the length of the homology region of your oligo and escape back to the top.

Positition the oligo window over the DNA sequence you want to use as the upper (left) primer by moving it with the home, end, and cursor up, down, right and left keys. If necessary use the go to (Position) option to move. Via the main (I) menu, then pick the current oligo as the upper oligo, 1. Note the melting temperature, Tm of your

oligo.

Do the same for the lower (right) primer, and enter this into the program as the lower oligo by entering 4. Also note the Tm of your oligo. Note that your oligo will be the bottom oligo shown in the pair at the bottom of the screen.

By pressing W you can see potential secondary structures in the two oligos, and from this submenu you can see potential dimers by pressing d. From the w menu you can also edit the sequence of your oligos, either the upper, u, or lower, l, primer.