Designing Footprinting Primers

Use the Oligo primer analysis software to design primers.
Primer 1 is farthest from the region of interest, and primer 3 is closest.
Primers 2 and 3 should have a 12-14 bp overlap.
Primers to footprint the coding strand are on the noncoding strand.
To design primers, Ping sets the nucleic acid concentrations to specific amounts (under the heading “non-search parameters”). He then looks for primers in an appropriate position with an appropriate TM. There are two ways to determine the TM in the Oligo program. One is by GC content. Ping does not use this way. The one he uses can be obtained by going under the heading “Window” and selecting “Current Oligo”. The TM and other information will then appear on the screen. Before selecting primers, Ping scans the sequence visually to make sure there aren’t too many repetitive bases or other weird things in the sequence of the primer.

Primer	Nucleic Acid Conc.	TM	Length	Position
1	10,000 pmole	62-63	about 23 bp	20-100 bp from primers 2 and 3
2	100,000 pmole	68-69	about 23 bp	70-90 bp away from region of interest
3	25,000 pmole	71-72	28-31 bp	overlaps primer 2 by 12-14 bp

Gel purify the primers.
Prepare the common linker

5’ GCG GTG ACC CGG GAG ATC TGA ATT C 3’
3’ C TAG ACT TAA G 5’

Gel purify each single stranded oligo

Anneal at a concentration of 20 mM in PCR machine

Store at -20 degrees C

The gel-purified longer oligo is also used separately in the PCR reaction with primer 2.

Genomic DNA Preparation for Suspension cells

Preparation:

Prepare area in hood for working with DMS:
Make about 200 mLs of a 3M solution of NaOH to put in a DMS waste container. All tips and tubes in contact with DMS go in here. The waste can later be emptied into a regular trash can after about a week or so.

Put 10 mLs of cell growth media into 50 mL Corning tube (1 tube per sample) and put in 37 degree water bath to warm.
Make 2 mLs of Lysis solution per sample
Lysis Stock: 50mM Tris, pH 8.0; 300 mM NaCl, 25 mM EDTA
store at RT
for Lysis Solution, add 0.2% f SDS and 0.2 mg/ml proteinase K to stock solution. Invert several times.
Make a 1 M solution of BME in cold PBS (need 40 mLs per sample. The BME stock is 14.4 M). Put solution on ice in hood.

Cell harvesting and DMS treatment:

Centrifuge 50 mLs of cells for 5 min. at 1000 rpm
Pour off supe.
Add 10 ul of DMS to 10 mLs of prewarmed media-swirl and add to cell pellet--mix. Swirl occasionally for 2 min at room temp.
Add 40 mL cold PBS/1 M BME and mix up and down.
Centrifuge for 5 min. at 1000 rpm.
Put supe in DMS waste.
Wash with 50 mLs cold PBS.
Centrifuge for 5 min at 1000 rpm.
Add 2 mL lysis buffer and put at 37 degrees for 3 hrs.

Can then leave at -20 degrees O/N if desired.

Phenol/ Cloroform extraction and Precip.:

Extract DNA 2X with phenol/ choroform/ isoamyl alcohol (25:24:1)

add 2.5 mLs and invert tube for 10 min by hand at med speed
spin in centrifuge for 10 min (1.5 K rpm for Beckman J2-HC).
transfer phenol layer into phenol waste with pasteur pipette
repeat

Extract DNA 2X with chloroform/ isoamyl alcohol (24:1)
same procedure as above

Transfer top layer to 15 mL Corning tube
Add 2.5X 100% EtOH and invert several times
-20 degrees for 2 hours to O/N
Spin down DNA 20 min/ 2000 RPM (~500G)
Remove supe with Pasteur pipette
Wash with 2 mLs of 75% ETOH (invert tubes to loosen pellet)
spin again for 20 min.
Dry in speed vac with caps on loosely for 1 min.
Resuspend in 200 ul of Gibco water
Transfer to lidlock ufuge tubes

<u>Piperidine Treatment:</u>

Add 20 ul Piperidine (stored in chrom. cabinet, 4 degrees)
Incubate at 90 degrees for 30 min.
Dry ice for 3 min.
Lyophilize without heat in speed vac (~ 2 hrs)
Resuspend in 150 ul water and repeat 2X.
EtOH precip. twice:
dissolve DNA in 400 ul water
add 40 ul 3M NaOAc, 10 ug tRNA, 1000 ul 100% EtOH
-20 degrees O/N

Spin 20 min., dissolve pellet in 400 ul water and precip. again
(no tRNA for second precip.)

Wash in 75% EtOH
Resuspend pellet in 50-75 ul of TE
Spin 10 min and transfer supe to autoclaved tube (removes debris)

<u>Determine Concentration:</u>

Take OD 260: dilute 1:1000 (1 ul in 1 mL of TE)
OD 260 x 40 = ug/ ul

Genomic DNA Preparation for Adherent Cells

<u>Cell treatment:</u>

Split cells evenly
Treat with cytokines if needed for appropriate time
Cells should grow to about 90% confluence before harvesting

Cell harvesting and DMS treatment:

Prepare area in hood for working with DMS:

Make about 200 mLs of a 3M solution of NaOH to put in a DMS waste container. All tips and tubes in contact with DMS go in here. The waste can later be emptied into a regular trash can after about a week or so.

Add about 100 g of solid NaOH into collection flask of aspirator that will be used.

UV treat cell scrapers

Put 10 mLs of cell growth media into 50 mL Corning tube (1 tube per sample) and put in 37 degree water bath to warm.

Make 2 mLs of Lysis solution per sample

Lysis Stock: 50mM Tris, pH 8.0; 300 mM NaCl, 25 mM EDTA
store at RT

for Lysis Solution, add 0.2% final of SDS and 0.2 mg/ml final of proteinase K to stock solution. Invert several times.

Make a 1 M solution of BME in cold PBS (need 5 mLs per sample. The BME stock is 14.4 M). Put solution on ice in hood.

Take first plate to hood and aspirate off media.

Add 10 ul of DMS to 10 mLs of prewarmed media-swirl and add to plate. Swirl occasionally for 2 min (Time with timer.)

Aspirate off into DMS waste flask.

Add 5 mL of 1M BME to plate, swirl for 10 sec, aspirate off into waste flask.

Wash plate with cold PBS 3X and aspirate.

Take plate to bench.

Add 1 mL lysis soln & swirl.

Scrape off goo with cell scrapers & transfer to 14 mL Falcon tube.

Add another mL and repeat.

Incubate tubes in 37 degree water bath with occasional swirling for 3-31/2 hours

Can then leave at -20 degrees O/N if desired.

Phenol/ Cloroform extraction and Precip.:

Extract DNA 2X with phenol/ choroform/ isoamyl alcohol (25:24:1)

add 2.5 mLs and invert tube for 10 min by hand at med speed

spin in centrifuge for 10 min (1.5 K rpm on Beckman J2-HC).

transfer phenol layer into phenol waste with pasteur pipette

repeat

Extract DNA 2X with chloroform/ isoamyl alcohol (24:1)

same procedure as above

Transfer top layer to 15 mL Corning tube

Add 2.5X 100% EtOH and invert several times

-20 degrees for 2 hours to O/N

Spin down DNA 20min/ 2000RPM (~500G)

Remove supe with Pasteur pipette

Wash with 2 mLs of 75% ETOH (invert tubes to loosen pellet)

spin again for 20 min.

Dry in speed vac with caps on loosely for 1 min.
Resuspend in 200 ul of Gibco water
Transfer to lidlock ufuge tubes

Piperidine Treatment:

Add 20 ul Piperidine in the hood (stored in chrom. cabinet, 4 degrees)
Incubate at 90 degrees in water bath for 30 min.
Dry ice for 3 min.
Lyophilize to dryness without heat in speed vac (~ 2 hrs)
Resuspend in 150 ul water and repeat 2X.
EtOH precip. twice:
    dissolve DNA in 400 ul water
    add 40 ul 3M NaOAc, 10 ug tRNA, 1000 ul 100% EtOH
    -20 degrees O/N
Spin 20 min., dissolve pellet in 400 ul water and precip. again
    (no tRNA for second precip.)
Wash in 75% EtOH
Resuspend pellet in 50-75 ul of TE
Spin 10 min and transfer supe to autoclaved tube (removes debris)

Determine Concentration:
Take OD 260: dilute 1:1000 (1 ul in 1 mL of TE)
OD 260 x 40 = ug/ ul

Preparation of in vitro genomic DNA

This DNA sample is needed to provide a ladder of all G's present in a sequence. Test samples are then compared with this sample to determine if a site is protected or hypersensitive.

Make the genomic DNA by the same procedure, except do not treat the cells with DMS. Instead, treat the DNA with DMS prior to the piperidine cleavage step.

After drying, resuspend genomic DNA in 175 ml of 1X TE.
Make a 1% DMS solution: 5 ml of DMS + 495 ml water
Add 25 ml of 1% DMS to the genomic DNA, vortex briefly, and incubate at RT for 2 min.
Add 50 ml cold DMS stop solution (1.5 M NaOAc, pH 7.0, 1 M BME, 10 mg tRNA) and 750 ml ethanol pre-cooled on dry ice. Put on dry ice immediately to stop the reaction. Keep on dry ice for 10 min., then centrifuge for 10 min at full speed in microfuge. Wash the pellet with 75% EtOH. Dissolve the DNA in 200 ml of Gibco water, and proceed with piperidine cleavage step.

In Vivo Footprinting

Day One: PCR reaction # 1 and ligation to linker duplex

Centrifuge genomic DNA to precipitate “junk”
(10 min, 4 degrees C, 14K rpm)

Label PCR tubes and add 2-3 µg genomic DNA in 5 µl total volume to each tube (use water to make up difference)

Prepare Basic G’mish to make Extension G’mish and Vent G’mish (make enough for N+1 reactions)

Basic G’mish

10X Vent Bfr	3.0
100 mM MgSO4	0.9
25 mM dNTP mix	0.24
water	20.61
	25 µl total

Vent G’mish

Basic G’mish	3.0
Vent enzyme	0.25 (add right before use)
	3.25 µl total

Extension G’mish

Basic G’mish	22.0
1 µM primer 1	0.3 (dilute to 1 µM right before use)
	22.3 µl total

Add 22 µl Extension G’mish to each PCR tube
Cap- vortex briefly-spin briefly

PCR # 1: (Ping’s files are #20-22, user #77, on left machine. Files 20 and 21 are never changed. File 22 is changed each time.)

File 20: 97 degrees C / 4 min
85 degrees hold --------- add 3.25 µl Vent G’mish

File 22: 95 degrees C / 1 min
59 degrees C / 30 min (Ping’s oligos always use 59 degrees)

76 degrees C / 10 min

While PCR reaction is running, make Dilution G'mish and Ligation G'mish

Dilution G'mish

5X Ligase Bfr	12.0	
1 M DTT	1.1	
10 µg/µl BSA	0.3	
water	16.6	
	30 µl total	

Ligation G'mish

I M DTT	0.27	
10 µg/µl BSA	0.08	
20 mM linker duplex	5.00	(Ping's "link 1-6" in PCR tubes)
T4 DNA ligase (1 U/µl)	3.00	
5X Ligase Bfr	3.00	
water	3.65	

After PCR reaction, transfer samples to ice immediately.

Add 30 µl dilution G'mish and 15 µl Ligation G'mish to each sample

Ligate at 16 degrees C overnight (> 16 H).

Day Two: PCR reactions #2 and 3 (labelling reaction)

Make Precipitation G'mish:

3M NaOAc, pH 7.0	9
10 mg/ml tRNA	1
	10 µl total

Add 10 µl Precipitation G'mish and 260 µl reagent alcohol to each sample

Place at -20 degrees > 30min and then at -70 degrees >30 min.

While DNA is precipitating, prepare Basic G'mish to make Extension G'mish and Vent G'mish for PCR number 2:

Basic G'mish

10X Vent Bfr	10.0	
100 mM MgSO4		3.0
25 mM dNTP mix		1.6
water	15.4	
	30 µl total	

Vent G'mish

Basic G'mish	2.5
water	2.5
Vent	1.0
	6.0 µl total

Extension G'mish

Basic G'mish	27.5	
primer # 2		10 pmoles
L-linker		10 pmoles (the longer SS oligo used to make the duplex linker)
	~28 µl total	

Centrifuge samples for 20 min. Wash with 350 µl 75% ETOH at room temp. Vortex until pellet is loosened and spin for 10 more min.

Dry pellets. Dissolve in 68 µl water, add 28 µl Extension G'mish and 80 ml mineral oil.

PCR # 2 (takes 2 1/2 H):

File 20: 97 degrees C / 4 min
85 degrees C / 10 min --------- add 6 µl Vent G'mish

File 21: 95 degrees C / 1 min
66 degrees C / 2 min (Ping's oligos always use 66 degrees)
76 degrees C / 3 min + 5 sec for each successive cycle

17 cycles, then link to file 22

File 22: 95 degrees C / 1 min
66 degrees C / 2 min
76 degrees C / 10 min

4 degree soak

While PCR is running, label primer #3 and make Labelling G'mish and Vent Stop G'mish

Label primer #3 and purify:

Kinase labelling reaction (enough for 6-8 samples)

Primer # 3	5 pmoles per sample	
water		

	5.2 ml total volume	
	-----boil 2 min, put on ice-----	
gamma 32 P- ATP	1.0	(our label is 150 mCi/ ml.)
10X Kinase Buffer	1.0	
0.1 M DTT	0.8	
Kinase	1.0	
	---	
	10 µl total	

Incubate at 37 degrees C for 1-2 hr. Check percent incorporation. Heat at 65 degrees to inactivate enzyme.

Purify labelled primer on Bio-RAD P-6 micro spin column

Determine volume of labelled primer required for Labelling G'mish, and add water to obtain this total volume.
Resuspend resin in column by inverting sharply several times.
Insert column into 2 mL collection tube and spin 2 min at full speed.
Transfer column to microfuge tube, add sample to column and spin 4 min at full speed.
Add sample to Labelling G'mish below.

Labelling G'mish

10X Vent Bfr	0.5	
100 mM MgSO4		0.15
25 mM dNTP mix		0.4
labelled primer #3		3.45
Vent	0.5	
	5.0 µl total	

Vent Stop G'mish

1 M Tris-HCL, pH 7.5		2.95
0.5 M EDTA, pH 8.0	2.36	
3 M NaOAc, pH 7.0	26.6	
10 µg/µl tRNA		1.0
water	260.0	
	295 ml total	

PCR # 3:

Take samples out of PCR machine. Put them back when machine reaches > 80 degrees C.

File 20: 97 degrees / 4 min
85 degrees / 10 min -------- add 5 µl Labelling G'mish

File 22: 95 degrees / 1 min
69 degrees / 2 min (Ping's oligos always use 69 degrees)
76 degrees / 10 min
2 cycles, then 4 degree soak.

Transfer samples to an eppendorf tube containing 295 µl of Vent Stop G'mish

Extract with 500 µl phenol/ chloroform (1:1)

Transfer to eppendorf tube containing 1 ml reagent alcohol. Precipitate 1 H to overnight.

Day Three: Run test sequencing gel

(This can also be done at the end of day 2.)

Centrifuge samples for 20 min, wash with 500 ml 75% ETOH, vortex, spin 10 more minutes, dry.

Dissolve pellet in 20 µl Sanger dye and load 4 µl on 6% sequencing gel.

Run the top dye to 7 cm from the bottom.

Transfer gel to piece of old film and cover with saran wrap.

Put on PhosphorImager.

Day Four: Run real gel

Develop PhosphorImage. By eye, determine how much of each sample should be loaded to make them even.

Run gel to appropriate distance to see region of interest clearly.

Fix and Dry.

Put on film without screen for up to 5 days.