July 30, 1997

10 sets of PCR primers flanking tetranucleotide repeat sequences were selected from the CHLC Human Screening set version 1 / Weber Set 6, and purchased from Research Genetics (Huntsville, Alabama). Their use in evaluation of microsatellite instbaility is outlined here.

Citations:

For Selective UV irradiation:
Jovanovic, A.S., Boynton, K.A. and Mutter, G.L. Uteri of women with endometrial carcinoma contain a histopathologic spectrum of monoclonal putative precancers, some with microsatellite instability. Cancer Res., 56:1917-1921, 1996.

For the Tetra Primers and use:
Mutter GL, Boynton KA, Faquin WC, Ruiz RE, Jovanovic AS. Allelotype mapping of unstable microsatellites establishes direct lineage continuity between endometrial precancers and cancer. Cancer Res 56:4483-4486, 1996.

For Parsimony Algorithm:
Mutter GL, Boynton KA, Faquin WC, Ruiz RE, Jovanovic AS. Allelotype mapping of unstable microsatellites establishes direct lineage continuity between endometrial precancers and cancer. Cancer Res 56:4483-4486, 1996.

PROTOCOL

General Comments:

The sensitivity of this technique can detect DNA from a single cell, making results erroneous if a reaction is contaminated. Always wear gloves. The amplified PCR products should be kept away from the areas and pipets used to prepare stocks and the pre-PCR reaction mixtures. Opening and closing tubes, as well as running a microfuge generates substantial aerosol that can contaminate a reaction. For this reason, all stocks (esp. oligo masters) should be handled in the hood using special PCR pipet set. This is essential for any PCR detection requiring more than 40 cycles.

DNA ISOLATION:

FRESH TISSUE:
Standard Bead Beater micro scale or grind up large scale isolation by Pro-K digestion and extraction cleanup is adequate. BE SURE DNA NEVER IS HEATED ABOVE 60'C, as this will denature it and prevent digestion. High salt extraction protocol doesn't work well for unknown reasons (as per Sklar lab).

PARAFFIN TISSUES:
Avoidance of HEATING ABOVE 70'C is essential, and makes usual Crum Chelex protocol useless. That suggested below is a composite of several methods, with a special cleanup at the end with EtBr.

CUTTING SECTIONS

1)Set up the microtome with disposable blades after carefully and completely wiping down the knife area. Best to wipe with xylene impregnated wipe to remove all traces of old paraffin.

Chill blocks to be cut by placing in freezer (-20'C) for 1 hour. Longer is OK.

2)Using GLOVES, mount the block and orient chuck so the the block face will cut evenly. orient in both the right-left axis and up-down axis.

3)Lightly face the block to remove loosely adherent contaminants from the surface. Using a clean Kimwipe, rub down block face with warm water. Rub ice cube on block face for 2-3 seconds (Repeat when needed).

4)Cut two 4um thick paraffin sections (floated on water) for H&E. This will be used to map area to be inked.

5)For UV-PCR cut 7um paraffin sections and pick up on regular glass slides (from the box, no extra washing). 1-2 cuts per slide, depending on face size. Important to not let sections sit long in water bath, or DNA will be leached out. Use four slides per isolation tube.

6)Cut "tube" sections for PCR at 10um thickness. Cut section, then pick up ribbon or rolled paraffin with clean wooden stick or eppendorf tips. Transfer to 1.5ml screw cap microfuge tube. Use 3 sections per tube. Only consider increasing the number of sections per tube if the area of tissue cut is very small (< 0.5 cm^2) Cap and label.

6)Re-clean, and repeat above for new block.

2)Bake slides overnight at 37-55'C, or 2-3 hours at 58-62'C.

UV IRRADIATION:
This is an optional step to isolate DNA from only a part of a histologic section.
1)Under dissecting scope delineate the area desired by circling it on the H+E stained coverslipped slide with a fine felt marker. Write date of UV irradiation on circled slide so that it serves as a record of what area was selected.

2)Prepare one 1.5 ml tube containing 0.5ml of Waterproof Black Opaque ink (Perfex# Corp.: 1-609-399-6767) for each case to be processed.

3)Under darkfield dissecting microscope apply ink with a 1-200ul bevelled pipet tip to areas from which DNA is desired. This is the same area previously delineated in the H+E slide.

4)When ink is dry, Place slides in Stratalinker 1800. Set dose at maximum energy (9999 x 100 ujoules/cm2) and expose, carefully measuring the time needed for completion. Then, use the exposure time option to expose the slides for an additional 19 times the measured interval. (Total exposure 20 x 9999 x 100 ujoules/cm2, or 20 joules/cm2). Expect about 1 1/2 hours total exposure time.

5)Use a straight edge single sided razor to scrape irradiated tissue section from the slide. This will usually curl and be easy to place in a screw-top 1.5ml centrifuge tube. Don't unecessarily scrape areas that are not inked, as these DNAs are not desired anyway.) Put scraped material from about 4 slides in each tube.

LYSIS

1)The paraffin section will usually fragment in the tube, and may fly out when opened. Tap tube to bring tissue fragments into the bottom. DO NOT centrifuge, as this will pack the paraffin fragments into an undigestible mass. If needed gently push down paraffin fragments using large end of 200ul pipet tip.

2)Add 300ul Lysis buffer per tube. AGITATE by vortexing vigorously for 5-10 seconds. Tap tube to bring tissue fragments and lysis buffer droplets into the bottom. DO NOT centrifuge, as this will pack the paraffin fragments into an undigestible mass.

Lysis/Solubilization Buffer for paraffin sections

Component	Stock	Final	Vol. for 5ml	10ml
Proteinase K	0mg/ml	200ug/ml	100ul	200ul
Tris pH 8.3	1M	50mM	250ul	500ul
Carrier Glycogen	20mg/ml**	100ug/ml	25ul	
Tween 20	50%*	0.5%	50	100
Calcium Chloride	0.1M	1.5mM	75	150
Water		to vol	4500ul	9050ul

*Tween is manufactured at 100%, but should be diluted with an equal volume of water to reduce viscosity and make pipetting easier.

**Carrier mussel glycogen: Sigma Cat#G1508 (alternate is Carrier RNA= 16s+28s e.coli rRNA (Boehringer Cat.#206938)

3)Warm to 61'C to melt wax, and mix vigorously on vortexer, and tap on counter to get all fragments under fluid level.

4)Incubate tubes at 60-61'C for two days. Best to use oven to provide even heat. Spike twice with additional Pro.K (every 12-24 hours). When spiking, add 6ul of 10mg/ml ProK to each 300ul tube. Mix again.

RAPID CLEANUP: This protocol is fine for paraffin DNA isolates to be amplified by tetra primers. DO NOT USE FOR AR PROTOCOL.

1)At the end of the digestion interval add 1 drop Chelex. Add 36ul of 5mg/ml EtBr (stock 5mg/ml in water). CAUTION: when adding EtBr, swirl pipet tip a bit to mix, and prevent precipitate. Mix well. Let sit for 5 minutes before proceeding with phenol cleanup.

2)Add 30ul of 3M Sodium Acetate. Mix.

3)Extract with an equal volume (300ul) of Phenol/Chloroform mix, vortex. Spin 5 minutes and remove aqueous phase to new tube. Extract with an equal volume

of chloroform, spin 5 minutes, transfer aqueous phase to new tube.

4)Precipitate by adding 2 volumes (800ul) of ice cold 100% ethanol. Incubate overnight at -20'C.

5)Spin PPT 20 minutes @4'C, decant ethanol. Decant supernatant without warming tubes. Best done by putting tubes in Stratacooler during tranfers.

6)Wash pellet with 60ul of cold 70% ethanol. Keep on ice when working at bench) 70% ethanol. Vortex briefly but vigorously from temporary storage on ice (or Stratacooler) enough to disaggregate pellet. If pellet doesn't disaggregate after 1-2 minutes of vortexing, this is ok and you should stop trying to break it up. Spin 10 minutes at 4'C, dry pellet. Decant and dry briefly.

FULL CLEANUP

For the real connoisseur, this generates DNA of high purity for delicate enzymatic manipulations, at an efficiency reduced from the preceeding. Since no boiling is used, isolated DNA wil be double stranded and amenable to restriction digestion.

1)At the end of the digestion interval add 2 ul of 0.5M EDTA (final about 3mM EDTA) to chelate Ca++. This will chelate the Ca++, and prevent it from interfering with phenol extraction. Vortex vigorously. Let sit for 1-2 minutes before proceeding with phenol cleanup.

2)Extract with an equal volume (300ul) of Phenol, vortex and let sit for 1-2 minutes. This will break up the ink. Remove auqeous phase to new tube. Repeat once more with another 300ul phenol (Total of two phenol extractions).

3)Add 36ul of EtBr (stock 5mg/ml in water). Mix well. Let sit for 1-2 minutes. Mix again. CAUTION: when adding EtBr, swirl pipet tip a bit to mix, and prevent precipitate.

4)Add 1/10 volume (35ul) of 3.0 M SODIUM Acetate. Mix well.

5)Extract with equal volumes (about 500ul) of the following:
Phenol/Chloroform x 1
Chloroform x 3

6)Remove aqueous phase to fresh tube.

7)Add 1 drop of reconstituted Chelex-100 to aqueous phase. Use 1 ml serological pipet. Vortex Gently. Let Stand 5 minutes, vortexing every few minutes.

8)Spin briefly, and remove aqueous phase to fresh tube, leaving Chelex beads behind. This is best done with a P200 tip which is too small a diameter to pick up beads. BE SURE NOT TO TRANSFER BEADS, AS THEY WILL inhibit any reactions to follow.

9)Add 2 volumes (800ul) of cold 100% ethanol.

10)Incubate overnight at -20'C (preferred, minimum of 6 hours at -20'C). Pellet DNA with 20 minute spin at 4'C in eppendorf microfuge. Decant supernatant without warming tubes. Best done by putting tubes in Stratacooler during tranfers.

11)Wash pellet in ice cold (from -20 freezer, keep on ice when working at bench) 70% ethanol. Vortex briefly but vigorously from temporary storage on ice (or Stratacooler) enough to disaggregate pellet. If pellet doesn't disaggregate after 1-2 minutes of vortexing, this is ok and you should stop trying to break it up. Re-precipitate pellet by spinning at 4'C for 10 minutes. Decant and dry briefly (residual ETHANOL INHIBITS digestion!!!).

SAMPLE RESUSPENSION FOR PCR

Paraffin DNA: DNA from one block in 30ul of 1x PCR buffer.
Fresh(frozen tissue) DNA: 4ug of DNA in 30ul of PCR buffer, as below.
ALWAYS re-precipitate DNA stored in aqueous form so it can be resuspended in optimal buffer.

PCR PRIMERS:

11 sets of PCR primers (concordance of primer set designations by locus/primer/laboratory identifier, are: D1S518/GATA7C01/T1.2; D2S1384/GATA52A04/T2.4; D2S1399/GGAA20G04/T2.3; D3S2387/GATA22G12/T3.1; D3S2459/GATA68D03/T3.4; D4S1627/GATA7D01/T4.1; D5S1505/GATA62A04/T5.2; D5S816/GATA2H09/T5.3; D8S1132/GATA26E03/T8.2; D21S1435/GATA49E01/T21.1) flanking tetranucleotide repeat sequences were selected from the CHLC Human Screening set version 1 / Weber Set 6, and purchased from Research Genetics (Huntsville, Alabama).

Single Use: use @ 0.1uM
Multiplex Use: use @ 0.1uM each

Choice 1:Good sensitivity and very low background. Primer 8.2 tends to be a bit fainter than 5.2. PREFERRED for large scale screens.

Choice 2:Combine Primers T3.1 with T5.2. Gives maximal sensitivity in detecting RER+ cancers, but primer 3.1 tends to be a little "dirty" with frequent artifactual bands which diminish specificity

(increases false positives.)

Table I: Pattern of locus involvement in endometrial cancers with microsatellite instability.

11 PCR primer pairs were used to amplify DNA from 69 archival endometrial cancers and their companion normal myometria, and novel alleles ("hits") scored when the tumor contained a different pattern of alleles than the matched myometrium. 23% (16/69) of tumors demonstrated altered alleles at two or more loci characteristic of microsatellite instability. The percentage of RER+ cases with involvement of each locus is shown in the Table.

Primer	% RER+ Tumors with Hits
T 1.2	56
T 2.3	50
T 2.4	38
T 3.1	88
T 3.4	44
T 4.1	50
T 5.2	75
T 5.3	25
T 7.2	13
T 8.2	69
T 21.1	38

SET UP PCR REACTION.

1) Determine the number of (50ul) reactions to be performed.

Standard Controls include:

a)Negative control: Complete reaction (with Taq, oligos) missing target template. Should yield no product.

b)Positive Control: 100ng of known good DNA

2) Change to a pair of CLEAN GLOVES and:

Make "Master Mix" for above reactions in a single tube. Mix two extra aliquots so pipetting errors won't cause shortage. Taq is expensive so don't overmix!

* USE CLEAN PCR PIPET SET FOR ALL PIPETTING OF THESE STOCKS****
These pipets should NEVER be used to pipet PCR REACTION PRODUCTS.

"Master Mix"

Component	final	Stock	Final for 50ul
PCR Buffer*	1x	10x	5.0
dNTP Mix@	1x	10x	5.0
OligoA+B	0.2+0.2uM	10uMa+10uMb	1.0*
32PdTTP#	0.06uM	12.5uM	0.25
Water			to volume
Taq	1.25U/5ul	5U/ul	0.25ul

*Primer Mix:
Mix equal volumes of primer A (20uM) and primer B (20uM) to yield mixture of primers A + B @ 10um each. Use 1ul of mix per 50ul reaction.

@dXTP mix:
as per reagent section at end of this protocol. Note [TTP] is less than that of other cold nucleotides.

32P-TTP Source:
ICN #33015H: 32P-TTP; 10mCi/ml, >600Ci/mMol, 16.7uM
(NEN alternative 32PdTTP is: NEG005A: 32P dTTP; 10mCi/ml, 800 Ci/mMol, 12.5uM

3)To above master mix add 1ul template DNA. Do not add more than 1ul of DNA per tube!

4)Aliquot 50ul of Master Mix into individual tubes labelled to receive template.

5) Overlay reaction mixture with 30ul of Mineral Oil.

6) Load onto PCR machine and run.

Profile for Tetra Primers

T1.2; T2.4; T2.3; T3.1; T3.4; T4.1; T5.2; T5.3; T8.2; T21.1

Temp.	Time	Cycles
95	4 min	}
55	45 secs	} 3 cycles
72	90 secs	}
95	30 secs	}
55	45 secs	} 26 (total 29 cycles) cycles paraffin DNA
72	90 secs	}
72	7 minutes	1
4	Hold	1

CAUTION: THESE MANIPULATIONS ARE "POST PCR"
PCR products can easily be spread around and contaminate genomic DNA or reagent stocks, causing failure of future runs. Treat PCR products as if they are infectious, and change disposable work surfaces/gloves frequently.

There is a "dirty" post-PCR 20ul Ranin pipettor under the gel boxes which can be used for these steps.

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7)Prepare sample for loading on the gel. Add 4.0ul of Bromophenol Blue/Xylene Cyanol/ Ficoll to an empty 0.5 ml tube. To this, add 20 of post-PCR reaction products. Mix by repeated aspiration/expelling of the 20 ul sample.

Note: Glycerol based loading buffers yield a curved band due to the curved meniscus formed in the well on loading.

8)Load 4ul of sample/lane on 8% polyacrylamide gel using FLAT TIPS.

To pour 100ml 8% polyacrylamide gel:

Compound	Stock	Vol. for 100ml
Acrylamide	30%	26.6ml
TBE	5x	20ml
Water		52ml
Am.Persulfate	10%	1ml
TEMED		20ul

Size Markers: Best is Phi-X digested with HaeIII: END LABELLED with 32P

Phi X; Hae III
1,353
1,078
872
603
310
281
271
234
194
118
72

8)Run Gel in 1x TBE at Room Temperature. AT 200-500 Volts.
Run Bromophenol blue off gel, and xylene cyanol to within 3inches of bottom. The xylene cyanol will run off gel before Tetra products.

Voltage	Estimated time
250V	Overnight + half a day
300V	Overnight (~18 hours)
600V	6-7 hours

9)Transfer gel to 3M paper (careful, RADIOACTIVE).

10)Dry gel in slab dryer.
11) Expose pre-flashed XAR film at -70'C.

REAGENTS

Acrylamide:

30% stock:
29g acrylamide, 1g bis-acrylamide, in 100ml water
mix and sterile filter
Store at 4'C in foil covered bottle.

Loading Buffer:

Uses Ficoll, reducing trailing effect.
Need FLAT TIPS to load
10x buffer, add 1/10 vol to sample to run

Compound	Stock	10x	Vol.Stock for 5ml 10x
Tris Base	solid	108g/l	0.540g
Boric Acid	solid	55g/l	0.275g
EDTA(MW372		0.5M	0.930g
Ficoll	solid	30%	1.500g
Xylene Cyanol		0.5%	0.250g
Br-Ph-Blue	solid	0.5%	0.250g

Alternate Loading Buffer:

00.5% Bromophenol blue
00.5% Xylene Cyanol
50.0% Glycerol
Water (DEP) to volume

Calcium Chloride Dihydrate; 100mM solution

MW 147.02

for 100ml of 100mM (0.1M) solution add 1.47g of CaCL2(dihydrate) to 100ml water.

Chelex 100, Biotechnology Grade: (Bio-Rad Cat.#143-2832)

Suspend in water and autoclave.
Check pH. Should be 8-9.

Note: if excessively alkaline, add 1/10 volume 1M Tris pH 8.0 to reduce pH to 8.0-8.5
(recheck with pH paper)

Store at RT as a slurry.

10x dNTP mix:
dATP, dTTP, dGTP, and dCTP, each at 2mM in stock purchased from
Pharmacia(#27-2035-01)in neutralized form at 100mM

Master stocks of neutralized dNTP's from pharmacia

Reagent	Vendor	Cat#	Conc	Amount
dATP	Pharmacia	27-2050-01	100mM	250ul
dTTP	Pharmacia	27-2080-01	100mM	250ul
dCTP	Pharmacia	27-2060-01	100mM	250ul
dGTP	Pharmacia		100mM	250ul

To Make 10x working Stock: 2mM each dNTP, 8mM total dNTP

Compound	Stock	Final	1 ml final	2ml final
dATP	100mM	2mM	20ul	40ul
dTTP*	100mM	0.5mM	5ul	10ul
dGTP	100mM	2mM	20ul	40ul
dCTP	100mM	2mM	0ul	40ul
Water			935ul	1870ul
Final Vol		to vol	1000ul	2000ul

Aliquot in 100ul volumes and store at -20'C
*For use with 32P TTP

Glycogen, carrier :
make up as 20mg/ml
Carrier mussel glycogen: Sigma Cat#G1508

Oligonucleotide Primers: Aliquot in HOOD
10 sets of PCR primers ordered from Research Genetics:
(concordance of primer set designations by locus/primer/laboratory identifier, are:
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D7S1824/GATA32C12/T7.2;
D8S1132/GATA26E03/T8.2;
D21S1435/GATA49E01/T21.1)

PCR Buffer, 10x Mix: Aliquot in Hood
Mix components in the listed order, and AUTOCLAVE to solubilize gelatin

before aliquoting into 1ml. tubes for storage at -20'C.

Compound	Stock	Final	vol.20ml.	vol.50ml
Water	--	to vol	17.5ml	44ml
Tris, pH 8.4	1M	100mM	2.0ml	5.0ml
KCl MW74.6	solid	500mM	0.7456g	1.864g
Gelatin*	solid	200ug/ml	4.0 mg	10.0 mg
MgCl2 $	1M	15mM	300 ul	750 ul

*Gelatin: Sigma Cat. # G-2500
$for primers ar-a/b final 1x [MgCl] is 1.5mM. Different for other primers.

Phenol:
Solid phenol crystals from Fluka in 500ml bottle.
Add about 300ml of 1.0M Tris, pH 8.0
Let sit a few hours to solubilize
Shake occasionally to equilibrate phases.
Decant aqueous phase and replace with another 150ml 1M pH8.0 Tris
Shake, re-equilibrate for 30min or more at RT.
Decant 1M Tris and Replace with TE, pH 8.0
Store at 4'C.
WARNINGS:
DISCARD IF TURNS YELLOW
ALWAYS USE AT ROOM TEMPERATURE

Proteinase K 10 mg/ml stock:
Boehringer Cat# 161519, 25mg.
(Pro-K supplied already saturated with Ca++. Ca++ needs to be in buffer to maintain activity under adverse conditions, but not for stock storage.)
Reconstitute in 2.5 ml of water, gives 10 mg Pro K /ml
Self-digest at 37'C for 30 minutes
Store at 4'C up to 4 weeks.

RNA, carrier :
16s+28s e.coli rRNA (Boehringer Cat.#206938)
Supplied as 4ug/ul solution

Sodium Acetate:
3M stock: MW 82.03
Dissolve 24.6 g of sodium acetate into final volume of 100ml water

Stratalinker 1800: Stratagene Cat#400071

254nm bulbs

TBE Buffer

5x Recipe:

	Final Volume	
	1 L	500ml
Tris BASE	54g	27g
Boric Acid	27.5g	13.75g
EDTA,0.5M	20ml	10.0ml
Water	to volume	

TE buffer:

10 mM Tris HCl, pH 8.0
1 mM EDTA, pH 8.0

Waterproof Black Opaque ink (Perfex# Corp.: 1-609-399-6767)

Perfex Corp.
PO Box 7
252 Asbury Av.
Ocean City, NJ
08226
$19.75/pint $5.20 2 oz

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D3S2459/GATA68D03/T3.4
D4S1627/GATA7D01/T4.1
D5S1505/GATA62A04/T5.2
D5S816/GATA2H09/T5.3
D7S1824/GATA32C12/T7.2
D8S1132/GATA26E03/T8.2
D21S1435/GATA49E01/T21.1