

I. Genomic double stranded DNA (portion of the CpG island of *FMRI*)

5' ... CGGATGCATTTGATTTCCCA **CGCCACTGAGTG** CACCTCTGCAGAAATGGGCGTTCTGGCCCTCGCGAGGCAGTGGACCTGTCCACCGCCCTTCAGCCTTCCCGCCCTCCACCAAGCCCGGCAC...3'
 3' ... GCCTACGTAAACTAAAGGGT **GCGTGACTCAC** GTGGAGACGTCTTTACCCTGCAAGACCCGGGAGCGCTCCGTACCGCTGGACAGTGGGGGAAGTCGGAAGGCGGGAGGTGGTTCGGCGCGTG...5'

II. Restriction digest

+ **DraIII restriction enzyme and incubation** (recognition sequence in yellow)

OH P
 5' ... TGCATTCGATTCACCA **GTGC** CACCTCTGCAGAAATGGGCGTTCTGGCCCTCGCGAGGCAGTGGACCTGTCCACCGCCCTTCAGCCTTCCCGCCCTCCACCAAGCCCGGCAC...3'
 3' ... ACGTAAACTAAAGGGT **ACTCAC** GTGGAGACGTCTTTACCCTGCAAGACCCGGGAGCGCTCCGTACCGCTGGACAGTGGGGGAAGTCGGAAGGCGGGAGGTGGTTCGGCGCGTG...5'
 P OH

III. Addition and ligation of hairpin linker

GA OH
 C GCATCGCTTGA -3'
 T GCTAGCGA -5'
 TC P

OH P
 5' **GTG** CACCTCTGCAGAAATGGGCGTTCTGGCCCTCGCGAGGCAGTGGACCTGTCCACCGCCCTTCAGCCTTCCCGCCCTCCACCAAGCCCGGCAC...3'
 3' - **ACTCAC** GTGGAGACGTCTTTACCCTGCAAGACCCGGGAGCGCTCCGTACCGCTGGACAGTGGGGGAAGTCGGAAGGCGGGAGGTGGTTCGGCGCGTG...5'
 OH

+ **T4 DNA ligase and incubation**

GA
 C GCATCGCTTGA **GTG** CACCTCTGCAGAAATGGGCGTTCTGGCCCTCGCGAGGCAGTGGACCTGTCCACCGCCCTTCAGCCTTCCCGCCCTCCACCAAGCCCGGCAC...3'
 T GCTAGCGA **ACTCAC** GTGGAGACGTCTTTACCCTGCAAGACCCGGGAGCGCTCCGTACCGCTGGACAGTGGGGGAAGTCGGAAGGCGGGAGGTGGTTCGGCGCGTG...5'
 TC

IV. Sodium bisulfite conversion of cytosine (but not methyl-cytosine) to **uracil**

AGUATUGUTTGA **GTGU** AUU T T C AGAAATGGGCGTTTGGUUU **CGCG** AGG U AGTGC GAU UGTUAU **CGUUU** TTUAGUUTT **UCGUUUU** TUAUUAAGUU **CGCGUAU** ...3'
 G
 (loss of significant base-pair complementarity due to bisulfite conversion)
 T
 T
 UCU **TAGCGA** AUUUAU **GTGGAGAU** GTU TTTA **UUUGCA** AAGAU **GGGACGGC** TUU GTUAU **UGCT** TGGAU **AGTGG** GGGAAAGTUGGAAGGCGGGAGGTGGTTGG **CGCG** GTG...5'

V. Right primer anneals during 1st round of PCR amplification

5' to 3' extension by polymerase Right Primer (example)
 <-- <-- **RCAAAAATAATTCAARCRATA**-5'

AGUATUGUTTGA **GTGU** AUU T T C AGAAATGGGCGTTTGGUUU **CGCG** AGG U AGTGC GAU UGTUAU **CGUUU** TTUAGUUTT **UCGUUUU** TUAUUAAGUU **CGCGUAU** ...3'
 G
 T
 T
 UCU **TAGCGA** AUUUAU **GTGGAGAU** GTU TTTA **UUUGCA** AAGAU **GGGACGGC** TUU GTUAU **UGCT** TGGAU **AGTGG** GGGAAAGTUGGAAGGCGGGAGGTGGTTGG **CGCG** GTG...5'

VI. Right primer extends to make complementary copy of top and bottom strands

TCATAACAAACTCATAAAAAACATCTTTACCCTGCAAAACCAAAA **CGCTCCATCACGCTAAACAATAGCAAAAAATCAAAAAARCAAAAAATAATTCAARCRATA** -5'
 C AGUATUGUTTGA **GTGU** AUU T T C AGAAATGGGCGTTTGGUUU **CGCG** AGG U AGTGC GAU UGTUAU **CGUUU** TTUAGUUTT **UCGUUUU** TUAUUAAGUU **CGCGUAU** ...3'
 A
 A
 T
 A
 UCU **TAGCGA** AUUUAU **GTGGAGAU** GTU TTTA **UUUGCA** AAGAU **GGGACGGC** TUU GTUAU **UGCT** TGGAU **AGTGG** GGGAAAGTUGGAAGGCGGGAGGTGGTTGG **CGCG** GTG...5'
 ACAATCACTTAAATACACCTCTACAAAAATAAACGTTCTAACCTTC **CGCA** AACAATA **CGACCTATCACCGCCCTTCAACCTTCCCGCCCTCCACCAAAACCGGCAC**...3'

VII. Left primer anneals during 2nd round of PCR amplification

TCATAACAAACTCATAAAAAACATCTTTACCCTGCAAAACCAAAA **CGCTCCATCACGCTAAACAATAGCAAAAAATCAAAAAARCAAAAAATAATTCAARCRATA** -5'
 C
 A
 A
 A

ACAATCACTTAAATACACCTCTACAAAAATAAACGTTCTAACCTTC **CGCA** AACAATA **CGACCTATCACCGCCCTTCAACCTTCCCGCCCTCCACCAAAACCGGCAC**...3'
 <-- <-- **GGAGGTGGTTGGGYGYGTG**-5'
 Left Primer (example)

VIII. Left primer extends

AGTATTGTTGAGTGATTTTTGTAGAAATGGGCGTTTGGTTTT **CGCG** AGGTACTGCGATTGTTATCGTTTTTTAGTTTTTTCTGTTTTTTATTAAGTTCGCGTAT-3'
 G TCATAACAAACTCATAAAAAACATCTTTACCCTGCAAAACCAAAA **CGCTCCATCACGCTAAACAATAGCAAAAAATCAAAAAARCAAAAAATAATTCAARCRATA** -5'
 C
 TA
 TA
 A
 T
 ACAATCACTTAAATACACCTCTACAAAAATAAACGTTCTAACCTTC **CGCA** AACAATA **CGACCTATCACCGCCCTTCAACCTTCCCGCCCTCCACCAAAACCGGCAC**...3'
 TGTAGTGAATTTATGTGGAGATGTTTTATTTGCAAGATTGGGAGCGCTTTGTTATCGTGGATAGTGGGGGAAGTGGGAAGGCG **GGAGGTGGTTGGGYGYGTG**-5'

PCR now proceeds in the conventional fashion