

Appendix

Appendix A

Inclusion criteria

Pediatric patients who present at Songklanagarind Hospital due to

A. Mental retardation

Mental retardation is herein defined as individuals with an intelligent quotient (IQ) less than 70 by Standford Binet or WISC (Wechsler Intelligence Scale for Children) assessments

B. Developmental delay, particularly delayed acquisition of language, in cases where an IQ score is not available.

Patients are performed history taking and physical examination by pediatricians, following the FXS checklist (Limprasert, et al., 2000)

1. Family history including mental retardation, learning difficulties and delayed development.
2. Long and narrow face is based on clinical impression of long jaw and high forehead.
3. Prominent ears are considered to be present when the angle of ears and face was approximately 90 degrees. Large ears are measured the long axis of the ears and compared to the standard scale using the 95 percentile as the threshold.
4. Attention deficit/ hyperactivity are scored according to the DSM-IV criteria.
5. Testicular volume is measured with an orchidometer as millimeter and compared to a modified standard scale.

Associated features, such as high arched palate, hypertelorism, hyperextensible joint, simian crease, flat feet, seizure, hypotonia and valvular heart disease were described.

Exclusion criteria

- Individuals with prenatal, perinatal and postnatal complications i.e. APGAR score < 5, postnatal seizure within 3 days of life.
- Individuals with previous history of CNS infection.
- Individuals with hypothyroidism.
- Individuals with deafness.
- Individuals that clinical compatible with other syndromes.

Appendix B

Protocol for DNA extraction

1. Pour 5-10 ml blood sample into sterile a sterile 15 ml conical tube
2. Add TE buffer up to 12-13 ml and centrifuge at 3,000 rpm for 10 min
3. Repeat step 2 for 3 times or until the supernatant has no hemoglobin color.
4. Add 760 µl of solution A into the cell pellet, and then transfer the mixture to a new 1.5 ml microcentrifuge tube.
5. Add 40 µl of 10% SDS, and gently break up the cell pellet.
6. Add 10 µl of 10 mg/ml Proteinase K (final 0.1 mg/ml).
7. Incubate at 37-45°C for 16-18 hours or until the cell pellet is lysed.
8. Add 1 volume of saturated phenol: chloroform (1:1).
9. Mix by invert tube up and down, and centrifuge at 13,000 rpm for 2 min.
10. Pipette the supernatant to a new 1.5 ml microcentrifuge tube (avoid the white protein).
11. Repeat step 9-10 for 2 times or until the white protein is disappeared. Be careful for phenol contamination.
12. Add 2 volumes of 100% cold ethanol into the supernatant and mix by invert tube up and down until the DNA is precipitated (white strand or globule).
13. Hook the DNA with a yellow tip or a hook glass, wash with 70% ethanol and air dry.
14. Resuspend the DNA in 200-500 µl of TE buffer and store at 4°C.

Protocol for PCR for CGG repeats

Polyacrylamide gel electrophoresis

1. Prepare 6% denatured polyacrylamide gel

Urea	63 g
10X TBE	15 ml
40% acrylamide	22.5 ml
Sterile H ₂ O	up to 150 ml

2. Prepare glass plates.

Long plate (16 cm x 23 cm) is treated with anti-water solution [RAIN-AWAY (Wynn's), cleaned with 95% alcohol.

Short plate (16 cm x 20 cm) is cleaned with 95% alcohol.

Place the short glass plate onto the long glass plate.

3. Mix 30 ml of 6% acrylamide gel with 40 µl of TEMED and 80 µl of 25% ammonium persulfate.

4. Pour gel into a sandwich of glass plates (avoid a bubble and leakage).

5. Leave the sandwich of glass plates at room temperature for 30-60 min or until the gel is complete polymerization.

6. Preheat the sandwich of glass plates containing gel in 1X TBE buffer at 8 watts for 30 min in a vertical electrophoresis chamber.

7. Add 15 µl of loading buffer (95% v/v Formamide, 0.05% w/v Bromphenol blue, 0.05% w/v Xylene cyanol FF, 1 mM EDTA) into 10 µl of PCR product, boil for 5 min and immediately place it on ice.

8. Load 8 µl of the PCR product on gel and run at 8 watts for 2.5 hours or until Xylene cyanol FF dye run out of the gel.

Electroblotting transfer

1. Cut a 14 cm x 16 cm of positive charge nylon membrane (Hybond-N).

2. Cut a small piece at lower right corner of the membrane to orientate the blot.

Wet the membrane in 1X TBE.

3. Cut 3 pieces of 14 cm x 16 cm of 3 MM filter paper (Whatman).
4. Lift a long glass plate off, leave the gel adhere to the short glass plate
5. Place a dry 3 MM filter paper onto the gel.
6. Side short glass plate up and gently lift it off.

7. Place a wet membrane onto the gel, followed by 2 pieces of wet 3 MM filter paper onto the membrane. Side the sandwich up again and place a wet 3 MM filter paper onto it.

8. Place the sandwich onto semidry electroblotting equipment (Biorad) (the gel is on top the membrane). Run the equipment at 400 mA for 45 min.

9. Denature the blot with 0.4 N NaOH for 10 min and neutralize it in 2X SSC for 10 min.

10. Bake the blot at 80°C for 30 min.

Hybridization and chemiluminescent detection

1. Prepare wash buffers (wash A and wash B).

Wash A is made up from 20 ml of wash component A, 25 ml of wash component B and sterile H₂O up to 500 ml.

Wash B is made up from 2 ml of wash component A, 25 ml of wash component B and sterile H₂O up to 500 ml.

2. Preheat 10 ml of hybridization buffer, 40 ml of wash A and 30 ml of wash B at 56°C.

3. Place the blot into hybridization bottle. Prehybridize with 10 ml of wash A at 56 °C for 10 min.

4. Add 0.5 μl each of a (CGC) $_n$ probe and a probe for D1S80 allelic ladder into hybridization buffer, and then replace wash A with hybridization buffer containing probe.

5. Hybridize at 56°C for 20 min.

6. Wash with 30 ml of wash A at 56°C for 20 min.

7. Wash with 30 ml of wash B at 56°C for 20 min.

8. Place the blot into 50 ml of 1X Quick light buffer. Shake the blot at room temperature for 5 min.

9. Place the blot into a plastic bag, spray 2-3 puffs of Lumiphos-480 on the blot and seal the plastic bag.

10. Place the plastic bag onto an X-ray cassette and put an X-ray film on top (perform in the dark room).

11. Leave the X-ray cassette at 37°C for 1.5-2 hours (the time can be adjusted depend on the intensity of signal).

Protocol for Southern blot analysis

Genomic DNA digestion

Reagent	Volume (final concentration)
DNA	8-10 μg + H ₂ O up to 30 μl
5X reaction buffer	8 μl (1X)
0.1 M DTT	0.4 μl (0.01 M)
100 units/ μl <i>Eco</i> RI	1 μl (100 u)
50 units/ μl <i>Eag</i> I	1 μl (50 u)
Total	40 μl

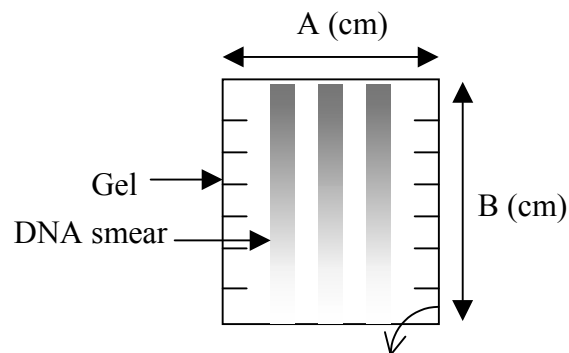
Digest at 37°C for 16-18 hours.

Electrophoresis of digested DNA

1. Prepare a 14 cm x 11cm x 0.6 cm of 0.8% (w/v) agarose gel in 1X TAE buffer.
2. Add 5 μ l of 10X Ficoll loading buffer (10X Ficoll loading buffer composes 25% Ficoll, 0.25% Xylene cyanol FF, 0.25% Brompheel blue) into the digested DNA.
3. Load the digested DNA on gel and run at 150 volts for 5 min, and then 30 volts until the bromphenol blue dye reaches the end of the gel (~17 hours).

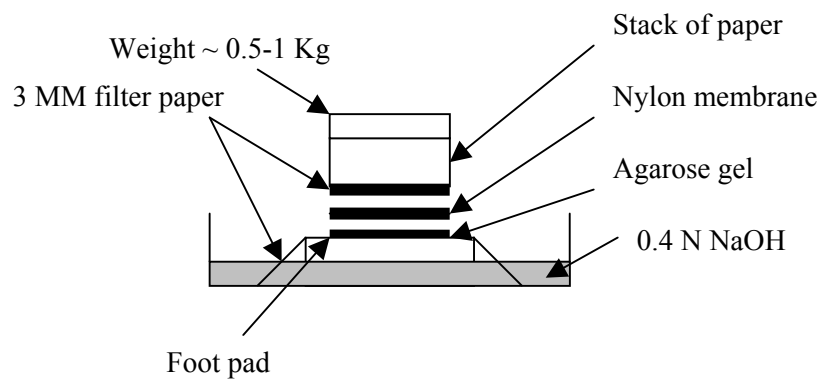
Southern transfer of DNA

1. Stain the gel with ethidium bromide, visualized under a UV transilluminator and photograph the gel.
2. Cut the gel at the upper edge of DNA smear and cut a small piece at the lower right corner to orientate the gel.
3. Cut A (cm) x B (cm) of nylon membrane and cut a small piece at the lower right corner for orientate the blot.



4. Cut 3 pieces of B (cm) x 25 cm of 3 MM filter paper and 3 pieces of A (cm) x B (cm) of 3 MM filter paper.
5. Prepare 500 ml of 0.4 N NaOH.
6. Put the gel onto 3 pieces of long 3MM filter papers. Leave both ends of the long 3 MM filter papers in 0.4 N NaOH. Put the membrane onto the gel. Avoid bubble

between the gel and the membrane. Put 3 pieces of short 3 MM filter papers onto the membrane. Put a stack of papers, followed by 0.5-1 Kg weight onto the 3 MM filter papers (see a figure below).



7. Leave at room temperature for 16-18 hours or until 0.4 N NaOH dry up.
8. Neutralize the membrane in 2X SSC for 10 min.
9. Bake the membrane at 80°C for 30-60 min.

Probe labeling

1. Dilute 5 μ l cross-linker solution with 80 μ l of supplied water to prepare a cross-linker working solution.
2. Dilute a StB12.3 probe with supplied water to a concentration of 10 ng/ μ l.
3. Place 10 μ l of diluted StB12.3 probe in a microcentrifuge tube, boil for 5 min, and immediately place it on ice for 5 min. Spin briefly.
5. Add 10 μ l of reaction buffer to the DNA. Mix thoroughly but gently.
6. Add 2 μ l of labeling reagent. Mix thoroughly but gently.
7. Add 10 μ l of the cross-linker working solution. Mix thoroughly. Spin briefly
8. Incubate the reaction at 37°C for 30 min.

The labeled-probe can be used immediately or kept on ice for up to 2 hours. For long term storage, may be stored in 50% (v/v) glycerol at -20°C for up to 6 months.

Hybridization and chemiluminescent detection

1. Prepare hybridization buffer.

Reagent	Volume (final concentration)
Hybridization buffer	100 ml
NaCl	2.925 g (0.5 M)
Blocking reagent	4 g (4% w/v)

Stir at room temperature for 1-2 hours. This buffer can be used immediately or stored in suitable aliquots at -20°C.

2. Preheat prehybridization buffer (primary wash buffer) and hybridization buffer at 56°C. The volume of hybridization buffer should be equivalent to 0.25 ml/cm² of the blot, but may be reduced to 0.125 ml/cm².

3. Place the blot into hybridization bottles. Add 10 ml of prehybridization buffer and hybridize at 56°C for 30-60 min.

4. Add the labeled probe into hybridization buffer (typically use 5-10 ng probe per 1 ml of buffer). Avoid placing the probe directly onto the blot.

5. Replace prehybridization buffer with hybridization buffer containing probe and hybridize at 56°C for 16-18 hours. Stringency can be changed by altering the hybridization temperature between 50°C and 75°C.

6. Prepare primary wash buffer. The primary wash buffer can be kept for up to 1 week in a refrigerator at 2-8°C.

Reagent	Volume (final concentration)
Urea	12 g (2 M)
10% SDS	1 ml (0.1%)
0.5 M NaH ₂ PO ₄ , pH 7.0	10 ml (50 mM)
1 M MgCl ₂	100 µl (1 mM)
Blocking reagent	0.2 g (0.2%)
NaCl (analytical grade)	0.87 g (150 mM)
H ₂ O	Up to 100 ml

7. Prepare secondary wash buffer. The secondary wash buffer can be kept for up to 4 months in a refrigerator at 2-8°C.

Reagent	Volume (final concentration)
Tris base	60.5 g (1 M)
NaCl (analytical grade)	56 g (2 M)
H ₂ O	Up to 500 ml after pH is adjusted to 10.0

8. Dilute secondary wash to 1:20 and add 2 ml/l of 1 M MgCl₂ (this working buffer should not be stored).

9. Preheat 1-2 ml/cm² of primary wash buffer at 56°C.

10. Replace hybridization buffer with primary wash buffer. Wash at 55°C for 10 min (2 times).

11. Place the blot in a clean container. Wash blot in 1-2 ml/ cm² of secondary wash-working buffer at room temperature for 5 min (2 times).
12. Transfer the blot into 1X Quick light buffer. Shake at room temperature for 5 min.
13. Place the blot into a plastic bag, spray 2-3 puffs of Lumiphos-480 on the blot and seal the plastic bag.
14. Place the plastic bag onto an X-ray cassette and put an X-ray film on top (performed in the dark room).
15. Leave the X-ray cassette at 37°C for 1.5-2 hours (time can be adjusted depend on the intensity of signal).

Protocol for Sodium bisulphite modification of DNA

1. Prepare 3 M sodium bisulphite, pH 5.0 and 10 mM hydroquinone
 - 3 M sodium bisulphite, pH 5.0
 - 7.52 g sodium bisulphite (Sigma)
 - 20 ml sterile H₂O
 - Adjust pH until 5.0 with 5 M NaOH (~1.2 ml)
 - 10 mM hydroquinone
 - 55 mg hydroquinone (Sigma)
 - 50 ml sterile H₂O
2. Dilute 2 µg of DNA with sterile H₂O up to 50 µl.
3. Add 5.5 µl of 3 M NaOH into DNA. Boil the DNA at 98 °C for 6 min under oil layer and immediately place it on ice (this step creates single-stranded DNA, **critical step**).
4. Add 30 µl of 10 mM freshly prepared hydroquinone and 520 µl of 3 M freshy

prepared sodium bisulphite. Assure that the reagents are mixed with DNA (hydroquinone is an antioxidant which prevents oxidation of intermediates formed during bisulphite treatment).

5. Layer with a mineral oil. Incubate at 55 °C for 4-6 hours.

6. Remove the mineral oil.

7. Add 1 ml of DNA wizard clean up resin (Promega A7280) and mix by invert tube up and down for 8-10 min.

8. Pipette the solution into the syringe barrel. Insert the syringe plunger slowly and gently push the solution into the minicolumn.

9. Detach the syringe from the minicolumn and remove the plunger from the syringe. Reattach the syringe barrel to the minicolumn.

10. Pipette 2 ml of 80% isopropanol into the syringe. Re-insert the plunger and push the solution through the minicolumn.

11. Remove the syringe barrel and transfer the minicolumn to a 1.5 ml microcentrifuge tube. Centrifuge the minicolumn at 12,000 rpm in a microcentrifuge for 2 min to dry the resin.

12. Transfer the minicolumn to a new 1.5 ml microcentrifuge tube, then add 50 µl of 65-75°C sterile H₂O or TE buffer to the minicolumn and wait for 3 min (the warmed water elutes the modified DNA from the resin).

13. Centrifuge at 12,000 rpm for 2 min to elute the bound DNA.

14. Remove and discard the minicolumn.

15. Add 5.5 µl of 3 M NaOH into a microcentrifuge tube and wait for 10 min at room temperature (this completes the chemical conversion of cytosine to uracil).

16. Add 1 µl of 10 mg/ml glycogen as a carrier.

17. Add 17 μl of 10 M ammonium acetate and 3 volumes ice-cold 100% ethanol.
18. Freeze at -20°C , overnight (or -70°C , 30-60 min).
19. Centrifuge at 12,000 rpm for 25 min.
18. Wash the modified DNA with ice cold 70% ethanol and centrifuge at 12,000 rpm for 5 min.
20. Dry the modified DNA in the vacuum.
21. Resuspend the modified DNA in 25 μl of TE buffer and store at -70°C .

Appendix C

Troubleshooting guide

Southern blot analysis

Problem and Possible causes

1. High background

- Too high concentration of cross-linker
- Labeling reaction left for >30 min
- Too high probe concentration
- Too low temperature of oven
- Too long hybridization time
- Bacterial contamination of the detection reagents
- Too much reaction reagent left on blot
- Too long exposure to film

2. Spotty background

- Bacterial contamination in the wash buffers
- Wash/ hybridization containers is not clean

3. Patchy background

- Insufficient wash buffer used, blot sticking together
- Insufficient hybridization buffer
- Membrane damage
- Blot allowed drying out
- Air bubble may have been trapped

4. Low signal

- Too dilute initial unlabeled DNA (<10 ng/μl)
- Too high DNA salt concentrations
- Too old cross-linker working solution
- Less than 2 μl labeling reagent used
- Reaction time < 20 min
- Too low probe concentration
- Labeled probe denatured before used
- Too high Oven temperature
- Too short Hybridization time
- Incorrect hybridization buffer used
- Too short exposure to film
- Too high a stringency used

5. Low stringency

- Too low oven temperature
- Incorrect salt concentrations in the hybridization buffer
- Incorrect SDS concentrations in the wash buffer

Methylation Specific PCR

Problem and Possible causes

1. No PCR product

- Lack of some reagent in PCR mixture
- PCR condition is not optimized (annealing temperature, Mg²⁺ concentration)
- Pipetting problems
- Primer to template ratio is not optimum (i.e. too low DNA template)
- Primer to primer ratio is not optimum

- Wrong cycling parameter
- Enzyme is not active
- Presence of PCR inhibitors

2. Faint band of some product

- Primer to template ratio is not optimal
- Primer to primer ratio is not optimal

3. Present of *XIST* unmethylated PCR product in male

- Wrong DNA sample
- Contaminated DNA sample

Appendix D

Tris-EDTA (TE) buffer

1 M Tris-HCl, pH 8.0 10 mM

0.5 M EDTA 1 mM

Solution A

5 M NaCl 0.14 M

1 M Tris-HCl, pH 8.0 0.01 M

1 M MgCl₂ 1.5 mM

NP-40 0.1%

10X TBE

Tris base 10.8 % w/v

Boric acid 5.5 % w/v

EDTA 0.02 M

20X SSC

NaCl 3 M

Sodium citrate 0.3 M

50X TAE buffer

Tris base 24.2 % w/v

100% glacial acetic acid 5.7 % v/v

EDTA 0.05 M

5X reaction buffer

1 M Tris-HCl, pH8.0 0.5 M

5 M NaCl 0.5 M

1 M MgCl₂ 0.1 M

Publications

Publication

1. Charalsawadi, C., Sripo, T., and Limprasert, P. 2005. Multiplex methylation specific PCR analysis of fragile X syndrome: experience in Songklanagarind Hospital. J. Med. Assoc. Thai.

Proceeding

1. Charalsawadi, C., Sripo, T., and Limprasert, P. Multiplex methylation specific PCR analysis of fragile X syndrome. Oral presentation at The 20th Annual Academic Meeting. Faculty of Medicine, Prince of Songkla University. August 17-20, 2004. Hat Yai, Songkhla, Thailand.
2. Charalsawadi, C., Sripo, T., and Limprasert, P. Multiplex methylation specific PCR analysis of fragile X syndrome: experience at Songklanagarind Hospital, Southern Thailand. Poster presentation at 5th Human Genetics Organization (HUGO) Pacific Meeting & 6th Asia-Pacific Conference on Human Genetics. Biopolis, Singapore.