CHAPTER III

METHODOLOGY

1. Research Instruments

1. Pipette tip : 10 µl, 100 µl, 200 µl, 1,000 µl (Elkay, USA)
2. Microcentrifuge tube : 0.2 ml, 0.5 ml, 1.5 ml (Bio-RAD, Elkay, USA)
3. Polypropylene conical tube : 15 ml (Elkay, USA)
4. Beaker : 50 ml, 100 ml, 200ml, 500 ml, 1,000 ml (Pyrex)
5. Flask : 250 ml, 500 ml, 1,000 ml (Pyrex)
6. Reagent bottle : 100 ml, 250 ml, 500 ml, 1,000 ml (Duran, USA)
7. Cylinder : 25 ml, 50 ml, 100 ml, 250 ml, 500 ml, 1,000 ml (Witeg, Germany)

Glass pipette : 5 ml, 10 ml (Witeg, Germany)

8. Pipette rack (Autopack, USA)
9. Thermometer (Precision, Germany)
10. Parafilm (American National Can, USA)
11. Cryo 1°C Freezing container (Nalgene® Labware)
12. Stirring-magnetic bar
13. Combs
14. Automatic adjustable micropipette : P2 (0.1-2 µl), P10 (0.5-10 µl), P20 (5-20 µl), P100 (20-100 µl), P1000 (0.1-1 ml) (Gilson, France)
15. Pipette boy (Tecnomara, Switzerland)
16. Vortex (Scientific Industry, USA)
17. pH meter (Eutech Cybernatics)
18. Stirring hot plate (Bamstead/Thermolyne, USA)
19. Balance (Precisa, Switzerland)
20. Centrifuge (J.P.Selecta, Spain)
21. Microcentrifuge (Eppendorf, Germany)
22. Mastercycler personal (Eppendorf, Germany)
23. Thermal cycler (Touch Down, Hybraid USA)
24. Power supply model 250 (Gibco BRL, Scotland)
25. Power poc 3000 (Bio-RAD)

26. Horizon 11-14 (Gibco BRL, Scotland)
27. Sequi-gen sequencing cell (Bio-RAD)
28. Heat block (Bockel)
29. Incubator (Memmert)
30. Thermostat shaking-water bath (Heto, Denmark)
31. Spectronic spectrophotometers (Genesys5, Milon Roy, USA)
32. UV Transilluminator (Fotodyne, USA)
33. UV-absorbing face shield (Spectronic, USA)
34. Gel doc 1000 (Bio-RAD)
35. Refrigerator 4°C (Misubishi, Japan)
36. Deep freeze -20°C, -80°C (Revco)
37. Water purification equipment (Water pro Ps, Labconco, USA)
38. Water bath (J.P.Selecta, Spain)
39. Storm 840 and ImageQuaNT solfware (Molecular dynamics)
40. 12-well culture plates (Corning, New York)
41. T-25 and T-75 Flasks (Corning, New York)
42. Costar® Stirpipette®: 0.2 ml, 10 ml, 25 ml (Corning, New York)
43. Haematocytometer counting chamber
44. Petridish (Sterilin limited, UK)
45. Cryotube vial 2.0 ml (Corning, New York)

2. Reagents

1. General reagents

1.1 Absolute ethanol (Merck)

1.2 Agarose, molecular grade (Promega)

1.3 Ammonium acetate (Merck)

1.4 Boric acid (Merck)

1.5 Bromphenol blue (Pharmacia)

1.6 Disodium ethylenediamine tetracetic acid : EDTA (Merck)

1.7 Ethidium bromide (Gibco BRL)

1.8 Ficoll 400 (Pharmacia)

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1.9 Hydrochloric acid (Merck)

1.10 Mineral oil (Sigma)

1.11 Phenol (Sigma)

1.12 Chloroform (Merck)

1.13 Isoamyl alcohol (Merck)

1.14 Sodium chloride (Merck)

1.15 Sodium dodecyl sulfate (Sigma)

1.16 Sodium hydroxide (Merck)

1.17 Sucrose (BDH)

1.18 Tris base (USB)

1.19 Triton X-100 (Pharmacia)

1.20 100 base pair DNA ladder (Biolabs)

1.21 40% acrylamide/bis solution 19:1 (Bio-RAD)

1.22 GelStar (Camberx)

2. PCR reagents

2.1 10X PCR buffer with KCl (500 mM KCl, 100 mM Tris-HCl pH 8.8, 0.8% Nonidet P40) (Fermentas)

2.2 10X PCR buffer with $(NH_4)_2SO_4$ (200 mM $(NH_4)_2SO_4$, 750 mM Tris-HCl pH 8.8, 0.1% Tween20) (Fermentas)

2.3 10X PCR buffer with $(NH_4)_2SO_4$ (200 mM $(NH_4)_2SO_4$, 750 mM Tris-HCl pH 8.8, 0.1% Tween20) (Immulase)

2.4 Magnesium chloride (Fermentas)

2.5 Magnesium chloride (Immulase)

2.6 Deoxynucleotide triphosphates (dNTPs) (Fermentas)

2.7 Oligonucleotide primers (BSU)

2.8 Oligonucleotide primers (Operon)

2.9 Oligonucleotide primers (BioDesign)

2.10 *Taq* DNA polymerase (Fermentas)

2.11 *Taq* DNA polymerase (Immulase)

2.12 100% DMSO

2.13 Genomic DNA sample

3. Restriction enzymes

    3.1 *EcoR*I (Biolabs)

    3.2 *Mbo*II (Biolabs)

    3.3 *Dpn*I (Biolabs)

4. Bacterial culture media

    4.1 Yeast extract powder (Bio Basic Inc.)

    4.2 Agar bacterial powder (Conda, Spain)

    4.3 Tryptone powder (Bio Basic Inc., Canada)

    4.4 Sodium chloride (BDH AnalaR®, Merck group)

5. Cell culture

    5.1 Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone)

    5.2 Fetal Bovine Serum (FBS) (GIBCO, Invitrogen)

    5.3 PenStrep (GIBCO, Invitrogen)

    5.4 Trypsin-EDTA (GIBCO, Invitrogen)

    5.5 Phosphate-buffered saline (PBS)

    5.6 Tryphan blue solution (Sigma)

6. Transfection reagents

    6.1 Lipofectamine™ 2000 (Invitrogen)

    6.2 Opti-MEMI Reduced Serum Medium (GIBCO, Invitrogen)

7. Bradford protein assay

    7.1 Bradford reagent (Bio-Rad)

    7.2 Bovine serum albumin; BSA (Sigma)

8. Ligation

    8.1 T4 DNA ligase

    8.2 5xT4 DNA ligase

9. Commercial Kits

    9.1 QIAamp® RNA Blood Mini Kit (QIAGEN)

    9.2 QIAamp® DNA Blood Mini Kit (QIAGEN)

    9.3 QIAquick® Gel Extraction Kit (QIAGEN)

    9.4 QIAprep® Spin Miniprep Kit (QIAGEN)

    9.5 QuiKChange® XL Site-directed Mutagenesis kit (Stratagene)

10. Alpha-L-iduronidase activity by fluorimetric assay

10.1 4-MU-alpha-L-iduronide (Glycosynth, Cheshire, United Kingdom)

10.2 Glycine (USB)

10.3 Sodium formate (Aldrich)

3. Experimental Procedure

3.1 Subjects and sample collection

3.1.1 Blood collection

After informed consent was received, approximate 5 ml. of peripheral blood from each individual was collected in a polypropylene tube with EDTA for RNA and DNA extraction.

Approximate 5 ml. of peripheral blood was collected in a polypropylene tube with heparin for an enzymatic assay in leukocytes.

3.1.2 Subjects

Two patients from unrelated families were clinically diagnosed with MPS I at the Pediatric Clinic of the King Chulalongkorn Memorial Hospital and were included in the study. Selection criteria were based on clinical presentation.

3.1.3 Controls

Controls were healthy volunteers unaffected with MPS I and had no family history of MPS I. DNA from the controls was used for mutation screening in the *IDUA* gene identified in the MPS I patients. DNA from the patients' family members who were at risk was also investigated.

3.2 Genetic analysis

3.2.1 DNA extraction

After informed consent, genomic DNA was isolated from peripheral blood leukocytes. This procedure was performed as follows:

1. 3 ml. of whole blood is centrifuged for 10 minutes at 3,000 rpm.

2. Remove supernatant and transfer buffy coat to a new polypropylene tube. Then add 10 volumes of cold lysis buffer 1 (or 10 ml.), mix thoroughly and incubate at –20°C for 5 minutes.

3. Centrifuge for 8 minutes at 13,400 rpm, and remove supernatant.

4. Add 3 ml. of cold lysis buffer 1, mix thoroughly and centrifuge for 8 minutes at 13,400 rpm.

5. Discard supernatant and add 900 μl of lysis buffer 2, 10 μl of proteinase K solution (20 mg of proteinase K in 1.0 ml. of 1% SDS-2 mM EDTA, and 50 μl. of 10% SDS). Mix vigorously for 15 seconds.

6. Incubate the tube(s) in a 37°C shaking waterbath overnight for complete digestion.

7. Add 1 ml. of phenol-chloroform-isoamyl alcohol and shake vigorously for 15 seconds and centrifuge at 6,000 rpm for 5 minutes.

8. Transfer the supernatant from each tube (containing DNA) to a new microcentrifuge tube.

9. Add 0.5 volumes of 7.5 M CH_3COONH_4 and 1 volume of 100% ethanol and mix by inversion. The DNA should immediately form a stringy precipitate. Recover the DNA by centrifugation at 13,400 rpm for 15 minutes. Then remove supernatant.

10. Rinse the pellet with 70% ethanol. Decant the ethanol and air-dry the pellet. (It is important to rinse well to remove any residual salt and phenol.)

11. Resuspend the DNA in 20-300 μl of the double distilled water at 37°C until dissolved.

3.2.1.1 Calculation of DNA concentration

The reading at 260 nm is used for calculating the DNA concentration. An OD of 1 corresponds to approximately 50 μg/ml for double-strand DNA. Therefore DNA concentration is calculated from the following:

$$\text{DNA concentration} = \text{OD} \times 50 \times \text{dilution ratio} (\mu g/ml)$$

3.2.2 RNA extraction

Total RNA was isolated from white blood cells using QIAamp® RNA blood mini kit (Qiagen, Valencia, CA). Reverse transcription was performed using ImProm-II™ reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instructions.

Procedure

1. Mix 1 volume of human whole blood with 5 volumes of Buffer EL in an appropriately sized tube.

2. Incubate for 10–15 minutes on ice. Mix by vortexing briefly 2 times during incubation.

3. Centrifuge at 4,000 rpm for 10 minutes at $4^{\circ}C$, and completely remove and discard supernatant.

4. Add Buffer EL to the cell pellet (use 2 volumes of Buffer EL per volume of whole blood used in step 1). Resuspend cells by vortexing briefly.

5. Centrifuge at 4,000 rpm for 10 minutes at $4^{\circ}C$, and completely remove and discard supernatant.

6. Add Buffer RLT to the pellet according to the table below. Vortex or pipet to mix.

7. Pipet lysate directly into a QIAshredder spin column in a 2 ml collection tube and centrifuge for 2 minutes at maximum speed to homogenize. Discard QIAshredder spin column and save homogenized lysate.

8. Add 1 volume (350 µl or 600 µl) of 70% ethanol to the homogenized lysate and mix by pipetting. Do not centrifuge.

9. Carefully pipet sample, including any precipitate which may have formed, into a new QIAamp spin column in a 2 ml collection tube without moistening the rim. Centrifuge for 15 seconds at 13,400 rpm. Maximum loading volume is 700 µl. If the volume of the sample exceeds 700 µl, successively load aliquots onto the QIAamp spin column and centrifuge as above.

10. Transfer the QIAamp spin column into a new 2 ml collection tube. Apply 700 µl of Buffer RW1 to the QIAamp spin column and centrifuge for 15 seconds at 13,400 rpm to wash.

11. Place the QIAamp spin column in a new 2 ml collection tube (provided). Pipet 500 µl of Buffer RPE into the QIAamp spin column and centrifuge for 15 seconds at 13,400 rpm.

12. Carefully open the QIAamp spin column and add 500 µl of Buffer RPE. And centrifuge at 13,400 rpm for 3 minutes.

13. Recommended: Place the QIAamp spin column in a new 2 ml collection tube and discard the old collection tube with the filtrate. Centrifuge at 13,400 rpm for 1 minute.

14. Transfer the QIAamp spin column into a 1.5 ml microcentrifuge tube (provided) and pipet 30–50 µl of RNase-free water (provided) directly onto the QIAamp membrane. Centrifuge for 1 min at 13,400 rpm to elute. Repeat if >0.5 ml whole blood (or >2 x 10^6 leukocytes) has been processed.

3.2.3 DNA amplification by Polymerase Chain Reaction (PCR)

Primer design guidelines:

- Primer length between 18-25 bp.
- Keep G-C content in the 30-80 % range.
- The Tm should be 55-60°C.
- G or C at the 3' end of primers will increase priming efficiency.
- Avoid runs of an identical nucleotide, especially guanine.
- Avoid secondary structure (hairpin, self-complementary and primer dimer).
- The five nucleotides at the 3' end should have no more than two G and/or C bases.
- Primer sequences should be searched using BLAST and checked for cross-homology.
- Primers should be specific with the target gene and not anneal with other genes.

3.2.3.1 Reverse Transcriptase - Polymerase Chain Reaction (RT- PCR)

For mutation screening, RT-PCR was initially performed.

RT-PCR Protocol

1. Mixture for cDNA preparation (reverse transcription)

Components	Amount
0.5 µg/µl Oligo dT Primer	1.0 µl (0.5 µg)
0.5 ng/µl RNA template	10.1 µl (5.05 ng)
Conditions	70°C, 5 minutes
	4°C, 5 minutes

2. Add the following components to the product of step 1

Components	Amount
5X Buffer	4.0 µl (1X)
25mM $MgCl_2$	2.4 µl (3.0 mM)
10mM dNTPs	1.0 µl (0.5 mM)
40U/µl RNasin® RNase Inhibitor	0.5 µl (20.0 U)
Reverse Transcriptase	1.0 µl
Total Volume	20.0 µl
Condition	25°C, 5 minutes
	40°C, 60 minutes
	40°C, 15 minutes

3. cDNA can be frozen for later use or used immediately for PCR. A sample without reverse transcriptase should be used as a control for each sample when preparing cDNA. This will check if there is DNA contamination in the RNA samples when doing PCR.

4. cDNA amplification: The primer pair used in RT-PCR (with the forward primer located at exon 3 and the reverse primer located at exon 11) is shown in Table 2.

Table 2 The primers used in RT-PCR.

Primer Name	Forward and Reverse primers (5'—>3')	Product size (bp)	Annealing Temperature (°C)
IDUA-F1	S : GGA CGG GTA CTT GGA CCT TC	1271	62
IDUA-R2	A/S : GCT GCC GTC GCT TTT GCT GG		

S = sense primer, A/S = antisense primer

Table 3 Mixture of RT-PCR.

Components	Amount
1. 10X PCR buffer	2.0 µl (1X)
2. 25mM $MgCl_2$	1.2 µl (1.5 mM)
3. 10mM dNTPs	0.4 µl (0.2 mM)
4. 10µM Forward primer	0.4 µl (0.2 µM)
5. 10µM Reverse primer	0.4 µl (0.2 µM)
6. 5U/µl *Taq* polymerase	0.1 µl (0.5 U)
7. Distilled water	11.5 µl
8. cDNA	3.0 µl
9. 100% DMSO	1.0 µl
Total volume (µl)	20.0 µl

Table 4 PCR conditions of cDNA amplification.

Step	Cycle	Temperature (°C)	Time
1. Initial denaturation	1	95	5 minutes
2. PCR - Denature	35	95	30 seconds
- Annealing		62	30 seconds
- Extension		72	2 minutes 30 seconds
3. Final extension	1	72	7 minutes

3.2.3.2 Polymerase Chain Reaction (PCR)

The other primers were designed within introns to allow genomic amplification and sequencing of exons 1-14 including exon-intron boundaries as shown in Table 5.

Table 5 Primers sequences for *IDUA* mutation analysis.

Exon	Primer Name	Primer sequences for PCR (5' to 3')	Product size (bp)	Annealing Temperature (^0C)
1	IDUA-Ex1F	S : ACC CAA CCC CTC CCA C	398	58
	IDUA-Ex1R	A/S : GCT CCG GTC TCT GAA GCT		
2	IDUA-Ex2F	S : GAA CGT GTG TGT CAG CCG	304	62
	IDUA-Ex2R	A/S : ACA AGG GGT CTT CCG AGC		
3-4	IDUA-Ex3/4F	S : TTC CAG CCT GGA GCA TGG AG	516	62
	IDUA-Ex3/4R	A/S : CTG CGT GAT AGG GGT GCA AC		
5-6	IDUA-Ex5/6F	S : TCA CCT TGC ACC CTC CCT CC	576	62
	IDUA-Ex5/6R	A/S : TCA GCA CCA CCA GGG TCA GC		
7	IDUA-Ex7F	S : TGC GGC TGG ACT ACA TCT C	448	62
	IDUA-Ex7R	A/S : AGT AGC AGG TTC TGA TGC TGC		
8	IDUA-Ex8F	S : CCA CCT TCC TCC CGA GAC	386	62
	IDUA-Ex8R	A/S : CTG GAG GAA GTG CGC TCC		
9-10	IDUA-Ex9F	S : TCC TTC ACC AAG GGG AGG	701	58
	IDUA-Ex10R	A/S : CCT GGA GAA CCC TGA GGA		
11-12	IDUA-Ex11/12F	S : GTG TGG GTG GGA GGT GGA	466	62
	IDUA-Ex11/12R	A/S : GTG ACC GCA TGG GTG AAG		
13-14	IDUA-Ex13/14F	S : CTG CCT GCT CCC ACC TTT GA	530	62
	IDUA-Ex13/14R	A/S : TGA TGG GAG GGC AGC ATG GG		

S = sense primer, A/S = antisense primer

Table 6 Mixture of PCR reactions.

Component	Volume per reaction (μl)				
	Exon 1	Exon 2	Exon 3-4	Exon 5-6	Exon 7
1. 10X PCR buffer with KCl	-	2.0 (1X)	2.0 (1X)	-	2.0 (1X)
10X PCR buffer with $(NH_4)_2SO_4$	2.0 (1X)	-	-	2.0 (1X)	-
2. 25mM $MgCl_2$	1.5 (1.8mM)	1.5 (1.8mM)	1.2 (1.5mM)	1.5 (1.8mM)	1.5 (1.8mM)
3. 10mM dNTPs	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)
4. 10μM Forward primer	0.4 (0.2μM)	0.4 (0.2μM)	0.4 (0.2μM)	0.4 (0.2μM)	0.4 (0.2μM)
5. 10μM Reverse primer	0.4 (0.2μM)	0.4 (0.2μM)	0.4 (0.2μM)	0.4 (0.2μM)	0.4 (0.2μM)
6. 5U/μl *Taq* polymerase	0.1 (0.5U)	0.1 (0.5U)	0.1 (0.5U)	0.1 (0.5U)	0.1 (0.5U)
7. Distilled water	11.2	13.5	13.5	12.2	12.2
8. 50ng/μl genomic DNA	2.0 (100ng)	2.0 (100ng)	2.0 (100ng)	2.0 (100ng)	2.0 (100ng)
9. 10%DMSO	2.0	2.0	-	1.0	1.0
Total volume (μl)	20.0	20.0	20.0	20.0	20.0

Component	Volume per reaction (μl)			
	Exon 8	Exon 9-10	Exon 11-12	Exon 13-14
1. 10X PCR buffer with KCl	2.0 (1X)	-	2.0 (1X)	2.0 (1X)
10X PCR buffer with $(NH_4)_2SO_4$	-	2.0 (1X)	-	-
2. 25mM $MgCl_2$	1.5 (1.8mM)	1.5 (1.8mM)	1.5 (1.8mM)	1.5 (1.8mM)
3. 10mM dNTPs	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)
4. 10μM Forward primer	0.4 (0.2μM)	0.4 (0.2μM)	0.4 (0.2μM)	0.4 (0.2μM)
5. 10μM Reverse primer	0.4 (0.2μM)	0.4 (0.2μM)	0.4 (0.2μM)	0.4 (0.2μM)
6. 5U/μl *Taq* polymerase	0.1 (0.5U)	0.1 (0.5U)	0.1 (0.5U)	0.1 (0.5U)
7. Distilled water	12.2	11.2	12.2	12.2
8. 50ng/μl genomic DNA	2.0 (100ng)	2.0 (100ng)	2.0 (100ng)	2.0 (100ng)
9. 10%DMSO	1.0	2.0	1.0	1.0
Total volume (μl)	20.0	20.0	20.0	20.0

Table 7 PCR cycle and conditions.

Step	Temperature and incubation time				
	Exon 1	Exon 2	Exon 3-4	Exon 5-6	Exon 7
1.Initial denaturation	95°C/ 5min	95°C/ 5min	95°C/ 5min	95°C/ 5min	95°C/ 5min
2.PCR cycle	35 cycles	35 cycles	35 cycles	35 cycles	35 cycles
- Denature	95°C/ 30sec	95°C/ 30sec	95°C/ 30sec	95°C/ 30sec	95°C/ 30sec
- Annealing	58°C/ 30sec	62°C/ 30sec	62°C/ 30sec	62°C/ 30sec	62°C/ 30sec
- Extension	72°C/ 30sec	72°C/ 30sec	72°C/ 30sec	72°C/ 30sec	72°C/ 30sec
3. Final extension	72°C/ 5min	72°C/ 5min	72°C/ 5min	72°C/ 5min	72°C/ 5min

Step	Temperature and incubation time			
	Exon 8	Exon 9-10	Exon 11-12	Exon 13-14
1.Initial denaturation	95°C/ 5min	95°C/ 5min	95°C/ 5min	95°C/ 5min
2.PCR cycle	35 cycles	35 cycles	35 cycles	35 cycles
- Denature	95°C/ 30sec	95°C/ 30sec	95°C/ 30sec	95°C/ 30sec
- Annealing	62°C/ 30sec	58°C/ 30sec	62°C/ 30sec	62°C/ 30sec
- Extension	72°C/ 30sec	72°C/ 30sec	72°C/ 30sec	72°C/ 30sec
3. Final extension	72°C/ 5min	72°C/ 5min	72°C/ 5min	72°C/ 5min

3.2.4 Direct sequencing

PCR products were treated with ExoSAP-IT (USP Corporation, Cleveland, OH), according to the manufacturer's recommendations, and sent for direct sequencing at the Macrogen Inc., Seoul, Korea. The sequence was analyzed using Sequencher (version 4.2; Gene Codes Corporation, Ann Arbor). When the result indicated a possible new variant, the sample was resequenced.

3.2.5 Agarose gel electrophoresis

The PCR products were run on a 1.0-1.5% agarose gel at 100 volts for 30 minutes. After electrophoresis, the DNA bands were stained with ethidium bromide and visualized by UV transillumination.

3.3 Enzyme assay

3.3.1 White blood cell (WBC) extraction

White blood cells are extracted by dextran extraction.

1. After centrifugation of heparinized whole blood, remove supernatant and add 0.85% NaCl to the tube with the total volume of 5 ml. Add 10 ml. of 3% dextran in 0.85% NaCl, then mix thoroughly by inverting the tube up and down. Let the tube stand for 30 minutes - 2 hours until phase separation.

2. Transfer the supernatant to a new tube, then centrifuge at 3,300 rpm for 10 minutes.

3. Remove supernatant and place the tube on ice.

4. Add 2 ml of 1.7% NaCl. After mixing, add 2 ml of cold distilled water and centrifuge at 3,300 rpm for 5 minutes.

5. Remove supernatant, add 1 ml. of 0.85% NaCl to the cell pellets.

6. Centrifuge at 3,300 rpm for 5 minutes. Remove supernatant and add 0.3-0.8 ml. of distilled water depending on the pellet size. The sample is then stored at -20°C before enzyme determination.

3.3.2 Determining the protein concentrations by Bradford protein assay.

The Bradford assay is rapid and accurate for the estimation of protein concentration. The Coomassie blue dye appears to bind most readily to arginyl and lysyl residues of proteins (not to the free amino acids). This specificity can lead to variation in the response of the assay to different proteins. Coomassie blue absorbs at 595 nm. It is sensitive to assay the proteins about 5 to 200 micrograms/ml.

The concentration of the protein sample is determined by comparison to that of a series of protein standards known to reproducibly exhibit a linear absorbance profile in this assay.

Reagents

1. Bradford reagent: Dissolve 100 mg of Coomassie Brilliant Blue G-250 in 50 ml. of 95% ethanol. Add 100 ml of 85% (w/v) phosphoric acid. Dilute with water to 1 liter. After the dye has completely dissolved, filter it through Whatman No.1 filter paper just before use and store it in an amber bottle at room temperature.

2. Protein standard (bovine serum albumin; BSA)

2.1 stock solution: 10.0 mg/ml

2.2 dilution: 1:2 dilution of stock solution - 5.0 mg/ml

1:4 dilution of stock solution - 2.5 mg/ml

1:8 dilution of stock solution - 1.25 mg/ml

1:16 dilution of stock solution - 0.625 mg/ml

1:32 dilution of stock solution - 0.3125 mg/ml

Assay

1. Dilution of samples to obtain between 10 and 100 µg of protein.

2. Pipet 6 µl of each sample and the standard solution into a clean and dry test tube. The measurement of protein concentration was performed in duplicate.

3. Add 300 µl of Bradford reagent to each tube, then mix well and leave on ice for 5 minutes.

4. At the end, read absorbance of each sample and the standard solution. Use 300 µl of Bradford reagent as a blank at 595 nm on spectrophotometer.

3.4 Functional analysis

3.4.1 Construction of plasmids

3.4.1.1 Construction of mammalian expression vectors

The wild-type *IDUA* cDNA (2,203-bp fragment) was cloned into the *Eco*RI restriction sites of the plasmid vector pEFNeo (received from National Health and Medical Research Council of Australia)[49] (Figure 4).

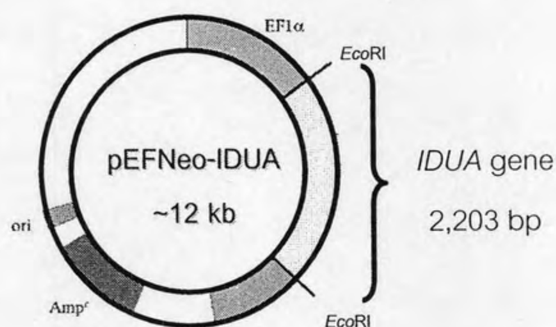

Figure 4 pEFNeo-IDUA expression vector.

3.4.1.2 Preparation of an empty vector

A control using the empty vector, digestion of pEFNeo-IDUA expression vector was performed with *EcoR*I. The digested fragment products were analyzed by electrophoresis on 1.0 % agarose gel stained with ethidium bromide along with the DNA marker and visualized by UV transillumination. Extraction of DNA fragment was performed using QIAquick®Gel Extraction Kit according to the manufacturer's instructions.

3.4.1.2.1 Procedure of gel extraction

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel. Minimize the size of the gel slice by removing extra agarose.

2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).

3. Incubate at 50°C for 10 minutes. To help dissolve gel, mix by vortexing the tube every 2–3 minutes during the incubation.

4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).

5. Add 1 gel volume of isopropanol to the sample and mix.

6. Place a QIAquick spin column in a provided 2 ml collection tube.

7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 minute.

8. Discard flow-through and place the QIAquick column back in the same collection tube. Collection tubes are re-used to reduce plastic waste.

9. Add 0.5 ml of Buffer QG to the QIAquick column and centrifuge for 1 minute.

11. Discard the flow-through and centrifuge the QIAquick column for an additional 1 minute at 13,400 rpm.

12. Place the QIAquick column into a clean 1.5 ml microcentrifuge tube.

13. To elute DNA, add 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or ddH_2O. Then centrifuge the column for 1 minute at 13,400 rpm.

3.4.1.2.2 Procedure of DNA ligation

Components of ligation mixture

T4 DNA ligase	2.0 µL
5X T4 DNA ligase buffer	3.0 µL
pEFNeo vector (300ng of vector)	5.0 µl
ddH_2O	5.0 µl

1. Set up the ligation mixture as shown above, invert the tube up and down to ensure thorough mixing.

2. Incubate the sample at 16°C overnight.

3. Transform bacteria with the ligation product and check for the correct size of the vector by restriction enzyme analysis using *EcoR*I.

3.4.2 Amplification of the expression vectors for transfection experiment

3.4.2.1 Preparation of competent cells

1. Grow bacteria (DH5α and XL-1 blue) from glycerol stock by streaking on the LB plate (without antibiotic) and incubate for 18 hours at 37°C.

2. Pick a single colony and grow them in 30 ml. of LB broth without antibiotic (starter media) in a 100-ml flask. Incubate the culture for 18 hours at 37°C with shaking at 225 rpm.

3. Dilute the starter media at ratio 1:10 with fresh LB broth without antibiotic (original media). Incubate the culture for 90 minutes at 37°C with shaking at 225 rpm.

4. Transfer 10 ml of the culture to a pre-chilled sterile 15-ml centrifuge tube. Pellet the bacteria with a 4,000 rpm spin for 10 minutes at 4°C. Discard supernatant and place the cell pellet on ice.

5. Resuspend cells in 10 ml. of cold 0.1 M $CaCl_2$ solution. Pellet the bacteria with a 4,000 rpm spin for 10 minutes at 4°C.

6. Discard supernatant and resuspend cells in 2 ml. of cold 0.1 M $CaCl_2$ solution per original media and add 10% glycerol. Mix by slowly pipetting up and down and store cells at –80°C.

7. Test for cell competency by transformation with the control plasmid vector using heat shock (see 4.2.2).

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3.4.2.2 Transformation

To make bacterial cells take up the plasmid/foreign DNA by using heat shock.

1. Take out competent (DH5α or XL-1 blue) cells from –80°C and thaw on ice for 5 minutes.

2. Add 5 μl of plasmid DNA into 50 μl of competent cells in a 1.5-ml microcentrifuge tube and gently stir with tip. Incubate for 30 min at –4°C.

3. Put tubes with DNA into heat block at 42°C for 45 seconds.

4. Put tubes back on ice for 2 minutes to reduce damage to the cells.

5. Add 1 ml of culture medium at 37°C containing SOC 980 μl, Mg^{2+} 10 μl, and 10 M glucose 10 μl (without antibiotic added). Incubate tubes for 90 minutes at 37°C with shaking at 225 rpm.

6. Spread 20-50 μl of culture by the spreader on warmed LB plates (with 100 mg/ μl of ampicillin). Grow them overnight at 37°C for 18 hours.

7. Pick a fresh single colony and place in 5 ml of LB broth (with 100mg/μl of ampicillin), and then incubate at 37°C with shaking at 225 rpm for 16 hours.

8. Extract and purify the plasmid DNA with mini prep.

3.4.2.3 Plasmid DNA extraction

The QIAprep® Spin Miniprep Kit was used according to the manufacturer's instructions.

1. Harvest bacteria from culture tubes into 1.5-ml microcentrifuge tubes. Centrifuge at 13,400 rpm for 3 minutes. Bacterial cells may be harvested in 15 ml tubes. Centrifuge at 5,400 rpm for 10 minutes at 4°C.

2. Discard supernatant and add 250 μl of chilled complete Qiagen suspension solution (P1), vortex or pipette up and down until no cell clumps remain. Transfer suspension cells to a 1.5 ml-microcentrifuge tube.

3. Add 250 μl of Qiagen lysis solution (P2) and mix thoroughly by inverting 10 times and let stand for 2 minutes at room temperature or until the lysate solution is clear.

4. Add 350 μl of Qiagen neutralize solution (N3) and mix thoroughly by inverting 10 times. Centrifuge for 10 minutes at 13,400 rpm in a table-top microcentrifuge. A compact white pellet will form.

5. Transfer the supernatant from step 4 to spin column tubes and centrifuge for 1 minute. Discard the flow-through.

6. Add 500 μl of Qiagen wash buffer (PB) and centrifuge for 1 minute. Discard the flow-through.

7. Remove all residual buffer PB by adding 750 μl of Qiagen wash buffer (PE) and centrifuge for 1 minute. Discard the flow-through and centrifuge again for 1 minute.

8. Transfer spin columns to a new 1.5-ml microcentrifuge tube, add 30-50 μl of filtered dH_2O or elution buffer (EB), and let stand for 5 minutes at room temperature. Centrifuge for 5 minutes at 13,400 rpm in a table-top microcentrifuge. Remove the column and store DNA at -20 ^{0}C.

3.4.2.4 DNA digestion

1. Obtain a buffer that works for all of the enzymes being used in the digestion. Vortex well and keep on ice.

2. Set-up a master mix consisting of the buffer (1, 2, 3, 4 or specific buffer for enzymes) that works for all enzymes being used, filtered dH_2O, and the enzymes. The digestion reaction was shown below.

3.4.2.4.1 Restriction enzyme digestion with *EcoR*I

*EcoR*I (20 U/μl)	0.5 μl
10XNEbuffer for *EcoR*I	2.0 μl
Distilled water	7.5 μl
DNA templates	10.0 μl
Total volume	20.0 μl

3. Incubate the reaction overnight in a 37 °C waterbath.

4. Check the sizes by electrophoresis of 20 μl of the digestion products on a 1.0 % agarose gel.

3.4.2.5 DNA precipitation

1. Add a half volume of 10 M NH_4OAc to 1 volume of the DNA sample.

2. Add an equal volume of chilled 100% EtOH to the DNA sample, then add 5 µl of glycogen, and mix thoroughly by gently inverting the tube up and down.

3. Incubate at –20°C overnight or at –80°C for 1 hour.

4. Centrifuge at 13,400 rpm for 15 minutes at 4°C and remove the ethanol.

5. Add 1 ml of chilled 70% EtOH, mix thoroughly by gently inverting, and centrifuge at 13,400 rpm for 5 minutes at 4°C.

6. Remove the ethanol with care and dry the pellet in a 50°C oven for 5 minutes or dry the pellet at room temperature overnight.

7. Resuspend the dried DNA in an appropriate amount of sterile TE (pH 8.0), or water, and store at 4°C for further manipulation or at –20°C for long-term storage.

3.4.3 Mutant strand synthesis

Two IDUA mutants including the one identified in our patient were selected for functional analysis. The mutant constructs were generated by *in vitro* site-directed mutagenesis (Stratagene's QuiKChange® XL Site-directed Mutagenesis kit) using the pEFNeo-IDUA as a template.

3.4.3.1 Mutant strand synthesis reaction (thermal cycling)

1. Synthesize two complimentary oligonucleotide primers containing the desired point mutation. The mutagenesis primer sets were designed by using Stratagene's web-based QuikChange® Primer Design Program available online at http://www.stratagene.com/qcprimerdesign. All mutagenesis primer sequences were shown in Table 8.

Primer Design Guidelines

The mutagenic oligonucleotide primers for this experiment must be designed individually for each desired mutation. The following considerations should be made for designing mutagenic primers:

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

1.2 Primers should be between 25 and 45 bases in length, with a melting temperature (Tm) of $\geq$ 78°C. Primers longer than 45 bases may be used, but using longer primers increases the likelihood of secondary structure formation, which may affect the efficiency of the mutagenesis reaction. The following formula is commonly used for estimating the T_m of primers:

$$T_m = 81.5 + 0.41(\%GC) - 675/N - \%\,mismatch$$

For calculating T_m:

- N is the primer length in bases.

- values for %GC and % mismatch are the numbers used for calculating T_m for primers intended to introduce insertions or deletions. The modified version of the above formula is used as follows:

$$T_m = 81.5 + 0.41(\%GC) - 675/N$$

where N does not include the bases which are being inserted or deleted.

Note: When using primer design software for QuikChange site directed mutagenesis applications, be aware that the T_m calculated by the primer design software may differ from the T_m value calculated using the formula presented above. Stratagene recommends verifying primer T_m's using the formula above or by using the QuikChange T_m calculator, available online at http://www.stratagene.com.

1.3 The desired mutation (deletion or insertion) should be in the middle of the primer with ~10–15 bases of correct sequence on both sides.

1.4 The primers optimally should have a minimum GC content of 40% and should terminate in one or more C or G bases

Table 8 Oligonucleotide primers for Quikchange site-directed mutagenesis of the *IDUA* cDNA

Mutation types	Mutagenesis primer sequences for PCR (5' to 3')	
p.E276K	S	: CCATCCTGGAGCAG**A**AGAAGGTCGTCGCG
	A/S	: CGCGACGACCTTCT**T**CTGCTCCAGGATGG
p.W402X*	S	: GGAGCAGCTCT**A**GGCCGAAGTGTCG
	A/S	: CGACACTTCGGCC**T**AGAGCTGCTCC

S = sense primer, A/S = antisense primer; the mutant nucleotide is bold and underlined.

* Oligonucleotide primers were designed by Beesley C.E. and others[50].

2. Prepare the sample reaction(s) as indicated in Table 9.

Table 9 Mixture of PCR reactions for site directed mutagenesis.

Components	Volume per reaction (µl)
1. 10X PCR reaction buffer	5.0 (1X)*
2. 50 ng/µl pEFNeo-IDUA	3.0 (150 ng)*
3. 125 ng Forward primer	1.25
4. 125 ng Reverse primer	1.25
5. dNTP mixture	1.0
6. QuickSolution	5.0
6. Distilled water	33.5
7. 2.5 U/µl *Pfu.Turbo* DNA polymerase	1.0
Total volume (µl)	51.0

*Final concentration per reaction in each PCR reaction

3. Cycle each reaction using the cycling outlined in Table 10.

Table 10 PCR cycle for site directed mutagenesis.

Step	Cycle	Temperature (°C)	Time
1. Initial denaturation	1	95	1 minute
2. PCR - Denature	18	95	50 seconds
- Annealing		60	50 seconds
- Extension		68	14 minutes
3. Final extension	1	68	7 minutes

3.4.3.2 *DpnI* digestion of the amplification products

This step was performed for digestion of the nonmutated parental DNA template with *DpnI*.

1. Add 1 μl of the *DpnI* restriction enzyme (10 U/μl) directly to each amplification reaction.

2. Gently and thoroughly mix each reaction mixture by pipetting the solution up and down several times. Spin down the reaction mixtures in a microcentrifuge for 1 minute and immediately incubate each reaction at 37°C for 1 hour to digest the parental DNA.

3.4.3.3 Transformation of competent cells

This step was performed for repairing the nicks in the mutated plasmid and for colony selection.

1. Gently thaw the XL10-Gold (XL1-Blue or DH5α) from -80°C on ice for 5 minutes. For each sample reaction, aliquot 50 μl of the supercompetent cells to a prechilled 1.5 ml microcentrifuge round-bottom tube.

2. Add 2 μl of β-ME to the 50 μl of supercompetent cells. Swirl and incubate the cells on ice for 10 minutes, swirling every 2 minutes.

3. Transfer 5 μl of the *DpnI*-treated DNA from each sample reaction to the 50 μl supercompetent cells and gently stir with tip. Incubate at –4°C for 20 minutes.

4. Put tubes with DNA into heat block at 42°C for 45 seconds.

5. Put tubes back on ice for 2 minutes to reduce damage to the cells.

6. Add 1 ml of medium at 37°C containing 980 μl of SOC medium, 10 μl of Mg^{2+}, and 10 μl of 10M glucose (without antibiotic added). Incubate tubes for 90 minutes at 37 °C with shaking at 225 rpm.

7. Plate the appropriate volume of each transformation reaction on agar plates containing the 100 mg/μl of ampicillin, 15 μl of 80 μg/ml X-gal and 4 μl of20 mM IPTG for colony selection.

8. Incubate the transformation plates at 37°C for 18 hours.

9. Pick a single colony and grow the cells in 5 ml LB broth (with 100 mg/ul of ampicillin), and then incubate at 37°C with shaking at 225 rpm for 16 hours.

10. Extract the plasmid DNA with Qiagen miniprep. (See plasmid DNA extraction protocol at 4.2.3)

11. Digest the plasmid DNA with *EcoR*I restriction enzyme and check for the correct size on ethidium bromide-stained 1.0 % agarose gel.

12. To check the products of *in vitro* site-directed mutagenesis for desired mutations, the plasmids were sent for direct sequencing.

3.4.4 Transfection assay

To test for an effect of a mutation on its protein Product (or alpha-L-iduronidase activity), a functional assay was described below.

3.4.4.1 Plating cells

This step was prepared for seeding cells in 6-well culture plates with DMEM containing 10% FBS (GIBCO) without antibiotics for 24 hours before transfection by Lipofectamine™ 2000 (Invitrogen). The number of cells for seeding was 6×10^5 cells/well.

1. Inspect COS7 cells (simian virus 40-transformed African green monkey kidney fibroblasts) in the T-75 flask by using an inverted microscope to ensure that they were 80-100% confluent.

2. Remove the growth medium from the T-75 flask, cells were then washed with 10 ml of 1X phosphate buffer saline (PBS).

3. Add 2 ml of Trypsin-EDTA and incubate cells in a 5% CO_2 incubator for 5 minutes.

4. Inhibit trypsinization with growth medium (DMEM+10% FBS) and subculture cells at a ratio of 1:3. Make the final volume to 10 ml of growth medium (DMEM+10% FBS).

5. Pipette up and down to mix cells, then transfer cells to a 15-ml centrifuge tube.

6. Use micropipette (size 1,000 μl) to mix cells again and sampling cells by pipetting 1 ml of suspension cells to a 1.5-ml microcentrifuge tube. Stain 50 μl of cells with 450 μl of trypan blue (cells:dye = 1:10).

7. Count cells using the hemocytometer by transferring cell solution into a counting chamber.

8. Calculate the number of cells by using formula as described below:

$$N \text{ (cell number per ml)} = \text{the average count per square} \times \text{dilution factor} \times 10^4$$

9. Seed cells with the number calculated (6×10^5 cells/well) in each well of the 6-well plate containing DMEM with 10% FBS (Hyclone) and incubate cells for 24 h in the CO_2 incubator.

Note: In this step, do not add antibiotics to the growth medium as this causes cell death.

3.4.4.2 Transfection

1. Prepare DNA for transfection.

pEFNeo-IDUA (wild-type or mutants)	4.0	µg/well
pEFNeo (empty vector)	4.0	µg/well

2. Dilute the working stock to 2.0 µg/µl of the plasmid DNA.

3. Dilute the plasmid DNA into 250 µl of Opti-MEM I reduced serum Medium without serum (GIBCO; Invitrogen), see Table 11.

Table 11 Mixture of transfection reaction for an assay of alpha-L-iduronidase activity

Components	Volume per reaction (µl)
*Plasmid DNA (pEFNeo-wtIDUA, pEFNeo-mtIDUA or pEFNeo empty vector)	2.0 µl
Opti-MEMI (µl)	248.0 µl
Total Voiume	250.0 µl

*wt = wild type, mt = mutant

4. Mix Lipofectamine™ 2000 gently before use, then dilute 10.0 µl of Lipofectamine™ 2000 in 240 µl of Opti-MEMI reduced serum medium. Incubate for 5 minutes at room temperature.

5. Combine the diluted DNA (from step 3) with the diluted Lipofectamine™ 2000 reagent (from step 4) (total volume = 500 µl). Incubate at room temperature for 20 minutes to allow DNA-Lipofectamine™ 2000 reagent complexes to form.

Note: Complexes are stable for 6 hours at room temperature.

6. Remove the growth medium from the well containing cells and add 1 ml of DMEM (without serum) and 500 µl of DNA-Lipofectamine™ 2000 complexes to each well. Add the DNA- Lipofectamine™ 2000 reagent complexes (500 µl) directly to each well (total volume = 2.0 ml/well) and mix gently by rocking the plate back and forth.

7. Incubate the cells at 37°C in a CO_2 incubator for 6 hours. Remove the transfection solution and replace with 1 ml of DMEM containing 10% FBS (Hyclone), 1% PenStrep, and incubate cells for 48 hours in a CO_2 incubator.

3.4.4.3 Prepare the cells for enzyme assay.

1. Remove the growth medium, then add 0.5 ml of Trypsin-EDTA and incubate cells in a 5% CO_2 incubator for 5 minutes.

2. Inhibit trypsinization with 1.0 ml of growth medium (DMEM+10% FBS) and transfer the cells to a labeled 1.5-ml microcentrifuge tube.

3. Centrifuge 300 rpm for 3 minutes at 4°C and remove supernatant.

4. Wash cells with 1.0 ml of 1x PBS.

5. Discard the supernatant and store cells at –80°C until the day of assay.

6. Before enzyme assay was performed, the cell lysate was prepared by using the sonicator.

7. All experiments were performed in triplicate and repeated two times. The levels of alpha-L-iduronidase activity were reported as mean ± SD.

3.4.5 Determining alpha-L-iduronidase activity by fluorimetric assay

The 4-MU-alpha-L-iduronide does not fluoresce unless being cleaved to release the fluorophore (4-MU). Fluorometric enzyme assays are based on the hydrolysis of 4-MU-containing substrates such as 4-MU-alpha-L-iduronide by alpha-L-iduronidase. Cleavage of 4-MU-alpha-L-iduronide by alpha-L-iduronidase yields the fluorescent molecule, 4-MU that emits light at 450 nm when excited by the 365-nm light.

Reagents

1. 1.0 nanomole/ml of 4-MU-alpha-L-iduronide

2. 0.1 mol/l of sodium formate buffer; pH 2.7

3. 0.5 mol/l of glycine-sodium buffer, pH 10.3

4. Fluorescent (4-Methylumbelliferone; 4-MU) standard solution (4-MU in glycine-sodium buffer)

5. stock solution: 10.0 nanomoles/ml

dilution 1:10 dilution of stock solution - 1.0 nanomole /ml

1:20 dilution of stock solution - 0.5 nanomole /ml

1:40 dilution of stock solution - 0.25 nanomole /ml

1:80 dilution of stock solution - 0.125 nanomole /ml

1:160 dilution of stock solution - 0.0625 nanomole /ml

1:320 dilution of stock solution - 0.03125 nanomole /ml

Assay

1. Set up the following tubes and run duplicate samples on the unknown specimens (Table 12).

Table 12 Mixture of enzyme assay.

	Sample (ml)	Cell blank (ml)	Standard (ml)
1.0 nanomole/ml of 4-MU-alpha-L-iduronide	0.04	-	0.04
Distilled water	-	0.04	0.04
0.1 mol/l of sodium formate buffer; pH 2.7	0.04	0.04	0.04
Cell lysate (50-120 ug)	0.04	0.04	-
Total volume	0.12	0.12	0.12

2. Immediately mix the above solution, and incubate for 1 hour in a 37°C water bath.

3. At the end of 1 hour incubation, stop the reaction with 2.88 ml of 0.5 mol/l glycine-sodium buffer, pH 10.3

4. Measure the absorbance of the sample with the fluorescence spectrophotometer (excitation and emission wavelength of 365 and 450 nm, respectively).

5. Calculate the enzyme activity in nmol/hour/mg protein.

6. Data analysis (using excel on the lab bench computers)

6.1 Calculate the enzyme activity (see 3.4.6)

6.2 The values are expressed as mean±SD and as percentage of the wild-type enzyme activity.

3.4.6 Calculation of enzyme activity

1. Determine the protein concentration by the method of Bradford protein assay. Results were given as mg protein per ml.

2. Subtract the readings of the substrate blank and the cell blank from that of the unknown sample to obtain a real value.

3. The formula for calculating enzyme activity was shown below.

$$\frac{\text{OD unknown}}{\text{OD std.}} \times \text{Conc. of std.} \times 3.0 \times 25.0 \times 1.0 \div \frac{\text{mg protein}}{\text{ml}}$$

Unit of enzyme activity in nmol/hour/mg protein; std = standard

When:

× 3.0 = correction factor for final volume

× 25.0 = correction factor for conversion of 40 μl of WBC or cell lysates used to 1,000 μl (1.0 ml)

× 1.0 = correction factor for 1-hour incubation period

3.4.7 Restriction enzymes digestion

Restriction enzyme digestion of the PCR products was performed with *Mbo*II. Digested and undigested PCR products were analyzed by electrophoresis on a 1.0 % agarose gel stained with ethidium bromide along with the DNA marker and visualized by UV transillumination.

3.4.7.1 Restriction enzyme digestion with *Mbo*II

*Mbo*II (5 U/μl)	0.5 μl
10XNEbuffer 2	2.0 μl
Distilled water	2.5 μl
PCR products	15.0 μl
Total volume	20.0 μl

Incubate the reaction mixture overnight at a 37°C waterbath.

3.4.8 The protein sequence is aligned with the ClustalX program.

3.4.8.1 Open ClustalX program version 2.0.11

After starting ClustalX, there is a window that looks something like the one below.

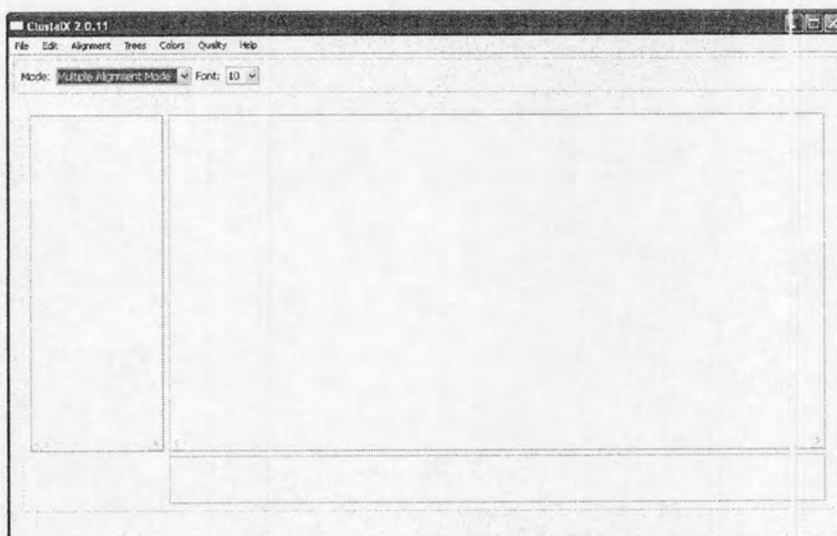

Figure 5 Show a window when starting the ClustalX program.

3.4.8.2 Read in the FASTA-formatted sequences

Pull down the File-menu, and choose Load Sequences menu item. Navigate to the folder (subdirectory) that contains the input file (text-file containing the sequences in FASTA format), and choose that file. Sequences should appear in the ClustalX window.

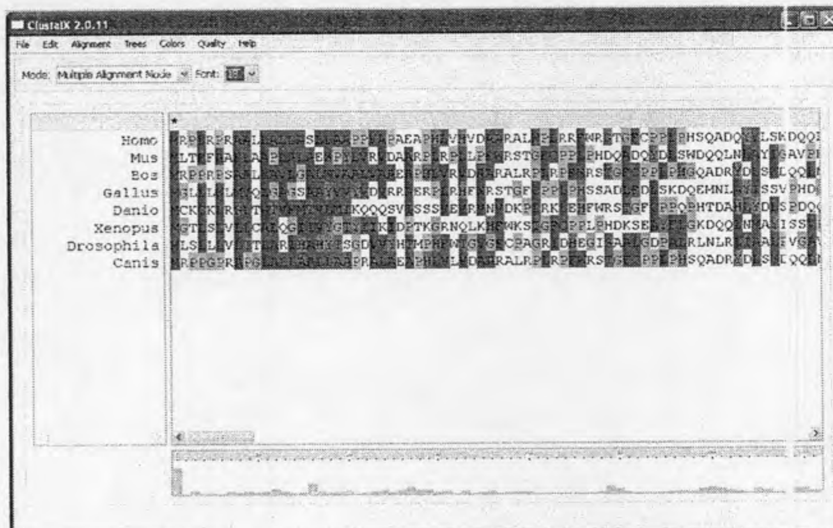

Figure 6 Display show protein sequences when pull down the File-menu and load sequence.

The left pane (in the figure above) lists the sequences according to the name that follows ">" symbol in the input file. The right pane shows the beginning of each sequence. You can scroll to the right to see the rest of each sequence by using the scroll bar at the bottom of the pane.

3.4.8.3 Modify the output format option, if necessary

Before aligning the sequences, you should make sure the output format options (from menu Alignment -> output format options) are set correctly. The Clustal formatted sequence alignment should be saved, An example of the output format option settings is shown in Figure 7.

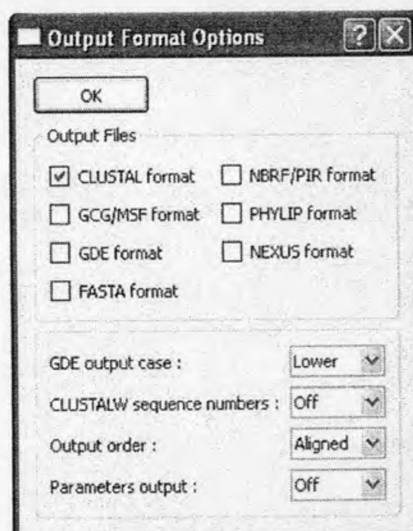

Figure 7 "Output format options" from the menu alignment.

3.4.8.4 Create an alignment

In order to make the actual alignment, select "Do complete alignment" from the menu Alignment. At that point ClustalX asks for output file names. Your sequence alignment is automatically saved in those files once the alignment is ready. After the alignment has been successfully calculated, a new view will appear. After the alignment has been created, ClustalX can be closed, and the generated alignment files in other programs can be used.

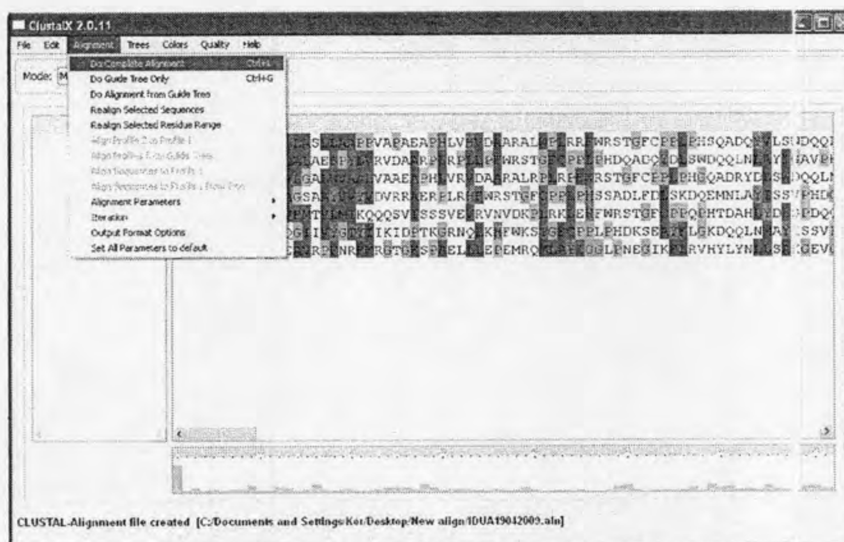

Figure 8 "Do complete alignment" from the menu alignment

3.4.8.5 The image above shows the main features of the ClustalX

1. The Menu Bar provides access to a range of different features and actions that can be applied to the alignment e.g. saving an alignment, adjusting the colouring scheme etc.

- In these instructions, menus and menu options are indicated using italics.

2. The Mode Drop-Down Box is used to switch between Multiple Alignment Mode (as shown above) and Profile Alignment Mode (see image below).

3. The Font Size Drop-Down Box is used to specify the size of the font used to represent the sequences in the Alignment Display Area.

4. The identifiers of the sequences shown in the alignment are displayed in the Sequence Identifier Panel.

5. The Consensus Line provides a summary of the degree of conservation of residues in the corresponding alignment column.

5.1 '*' (identical residues in all sequences)

5.2 ':' (highly conserved column)

5.3 '.' (weakly conserved column)

6. The Alignment Ruler indicates the position in the alignment (with the first column assigned position 1 etc.) of a given column shown in the Alignment Display Area.

7. The Alignment Conservation Display Area provides a bar chart indicating the degree of conservation of each column in the alignment.

Figure 9 Overview the main features of the ClustalX program.

3.4.8.6 Creating the input file for multiple sequence alignment

ClustalX is going to be used for sequence alignment. It, like any other computer program requires the data it manipulates (the input file) to be in a format it can recognize. The word processor can be used to create the input file. The unedited files look like this:

```
>gi|110611239|ref|NP_000194.2| alpha-L-iduronidase precursor [Homo sapiens]
MRPLRPRAALLALLASLLAAPPVAPAEAPHLVHVDAARALWPLRRFWRSTGFCPPLPHSQADQYVLSWDQ
QLNLAYVGAVPHRGIKQVRTHWLLELVTTRGSTGRGLSYNFTHLDGYLDLLRENQLLPGFELMGSASGHF
TDFEDKQQVFEWKDLVSSLARRYIGRYGLAHVSKWNFETWNEPDHHDFDNVSMTMQGFLNYYDACSEGLR
AASPALRLGGPGDSFHTPPRSPLSWGLLRHCHDGTNFFTGEAGVRLDYISLHRKGARSSISILEQEKVVA
QQIRQLFPKFADTPIYNDEADPLVGWSLPQPWRADVTYAAMVVKVIAQHQNLLLANTTSAFPYALLSNDN
AFLSYHPHPFAQRTLTARFQVNNTRPPHVQLLRKPVLTAMGLLALLDEEQLWAEVSQAGTVLDSNHTVGV
LASAHRPQGPADAWRAAVLIYASDDTRAHPNRSVAVTLRLRGVPPGPGLVYVTRYLDNGLCSPDGEWRRL
GRPVFPTAEQFRRMRAAEDPVAAAPRPLPAGGRLTLRPALRLPSLLLVHVCARPEKPPGQVTRLPALPLT
QGQLVLVWSDEHVGSKCLWTYEIQFSQDGKAYTPVSRKPSTFNLFVFSPDTGAVSGSYRVRALDYWARPG
PFSDPVPYLEVPVPRGPPSPGNP

>gi|6680349|ref|NP_032351.1| iduronidase, alpha-L- [Mus musculus]
MLTFFAAFLAAPLALAESPYLVRVDAARPLRPLLPFWRSTGFCPPLPHDQADQYDLSWDQQLNLAYIGAV
PHSGIEQVRIHWLLDLITARKSPGQGLMYNFTHLDAFLDLLMENQLLPGFELMGSPSGYFTDFDDKQQVF
EWKDLVSLLARRYIGRYGLTHVSKWNFETWNEPDHHDFDNVSMTTQGFLNYYDACSEGLRIASPTLKLGG
PGDSFHPLPRSPMCWSLLGHCANGTNFFTGEVGVRLDYISLHKKGAGSSIAILEQEMAVVEQVQQLFPEF
KDTPIYNDEADPLVGWSLPQPWRADVTYAALVVKVIAQHQNLLFANSSSSMRYVLLSNDNAFLSYHPYPF
SQRTLTARSQVNNTHPPHVQLLRKPVLTVMGLMALLDGEQLWAEVSKAGAVLDSNHTVGVLASTHHPEGS
AAGWSTTVLIYTSDDTHAHPNHSIPVTLRLRGVPPGLDLVYIVLYLDNQLSSPYSAWQHMGQPVPPSAEQ
FRRMRMVEDPVAEAPRPFPARGRLTLHRKLPVPSLLLVHVCTRPLKPPGQVSRLRALPLTHGQLILVWSD
ERVGSKCLWTYEIQFSQKGEEYAPINRRPSTFNLFVFSPDTAVVSGSYRVRALDYWARPGPFSDPVTYLD
VPAS
```

This line contains the title of the sequence. The sequence will start from the next line. The ">" character should be followed by one word (only letters or numbers and _), which ClustalX will use as the name for the sequence in the multiple alignment that it creates. Clustal treats everything between ">" and the first space as the sequence name.