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Diagnosis of Copper Transport Disorders

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Abstract

Techniques for the diagnosis of copper transport disorders are increasingly important due to recent recognition of previously unappreciated clinical phenotypes and emerging advances in the treatment of these conditions. Here, we collate the diagnostic approaches and techniques currently employed for biochemical and molecular assessment of at-risk individuals in whom abnormal copper metabolism is suspected.

Introduction

The protocols described here are used for biochemical or molecular diagnosis of copper transport disorders. Prenatal detection of Menkes disease may be performed by molecular analysis of *ATP7A* from amniocyte DNA, and is also possible by determination of chorionic villus copper levels (Kaler and Tümer, 1998). The latter test results from abnormal accumulation of placental copper in affected pregnancies. Postnatally, the diagnosis is possible from plasma catechol levels and ratios (Kaler et al., 1993; Goldstein et al, 2009) or whole blood DNA analysis. The former test relies on partial deficiency of dopamine-beta-hydroxylase in affected infants. Since Menkes disease is an X-linked condition, most of the affected patients are male.

The molecular diagnosis of Menkes disease (MIM 309400), Occipital Horn Syndrome (OHS) (MIM 304150), and *ATP7A*-related distal motor neuropathy (MIM 300489) is performed by identification of the disease causing mutation in the *ATP7A* gene (MIM 300011). Multiplex PCR can be performed to screen for large deletions. Screening for base-pair substitutions and small base pair deletions, duplications or insertions are performed by sequencing of the 23 exons including 20 base pair flanking sequences (splice junctions). Additional molecular screening for exon deletions or duplications can be performed using Multiplex Ligation-dependent Amplification (MLPA) (Schouten et al., 2002).

Approximately 95% of *ATP7A* mutations are identified by these techniques. In about 5% of the suspected patients, no mutation is identified, using any of these methods. A fraction of these mutations might be located in the 5'UTR, 3'UTR or intronic sequences that are not analyzed, or represent phenocopies due to alterations in other genes not yet appreciated.

If a family's specific mutation is identified, at-risk female carrier testing or prenatal diagnosis (using fetal genomic DNA extracted from CVS or amniotic fluid cells) can be performed by sequencing of the affected exon, by MLPA testing, or by screening for the presence of a junction fragment (in the case of large deletions) (Desai et al., 2010).

The Genbank sequence NM_000052 is used as reference sequence for *ATP7A* where A in the ATG start codon is numbered as c.1 and the ATG codon as p.1. For identified mutations see <http://www.hgmd.cf.ac.uk/ac/index.php>. Site registration is required and access is free for academic and non-profit organizations.

Mutations in *ATP7B* are responsible for Wilson disease (Schilsky, 2009; Kaler, 2010), an autosomal recessive condition. Mutations in the gene encoding ceruloplasmin (*CP*) cause Aceruloplasminemia. Mutations in *CCS*, the chaperone to copper superoxide dismutase, may disturb copper homeostasis (Huppke et al., 2010). Other genes to consider screening in patients presenting with low serum copper and ceruloplasmin include *CTR1*, which encodes a high affinity copper uptake protein, and *ATOX1*, the copper chaperone to *ATP7A* and *ATP7B*. No reports of *CTR1* or *ATOX1* mutations have been reported to date. Elevated serum copper and ceruloplasmin levels have been reported in patients with idiopathic copper toxicosis (Hahn et al., 1994), although no clear genetic basis for this entity has been delineated.

Basic Protocol 1: Plasma Catechol Patterns to Diagnose Menkes Disease

Biochemical methods are often superior for rapid diagnosis when there is clinical suspicion of disease, and molecular techniques used for confirmation. In this context, neurochemical analysis of the plasma catechols, dihydroxyphenylglycol (DHPG), norepinephrine (NE), dihydroxyphenylalanine (DOPA), dopamine (DA), and dihydroxyphenylacetic acid (DOPAC) by high-pressure liquid chromatography with electrochemical detection (HPLC-EC) has become an invaluable rapid method for diagnosing Menkes disease in newborns (Figure 1). By measuring ratios of DOPA:DHPG, DOPAC:DHPG, and DA:NE, newborns suspected of Menkes disease can be diagnosed quickly.

Because dopamine-beta-hydroxylase (DBH), an important enzyme in catecholamine synthesis, is copper dependent, and Menkes disease patients have an impairment in incorporation of copper, catechols that occur in the catechol pathway prior to DBH should be high, and those after should be low.

Overview

Plasma samples are extracted via a batch alumina extraction, the alumina eluate is injected into an HPLC-EC system, and the concentrations of catechols are calculated against an extracted mixture of standards of known concentrations. The alumina extraction efficiently separates the catechols from other much more abundant compounds in plasma, greatly improving assay sensitivity and specificity.

Materials

Chemicals

- Sodium phosphate, monobasic
- Ethylenediamine-tetraacetic acid, disodium salt (EDTA)
- Octane sulfonic acid
- Ortho-phosphoric acid-HPLC grade

Hydrochloric acid
Acetic acid
Acetonitrile-HPLC grade
TRIS base, ULTRA Pure
Alumina, Acid washed, Activity grade I

Analytical column

Axxi-chrom 5 μ m 4.6 $\times$ 250 mm C18 reversed phase (Axxion)

Standards

DL-3,4-Dihydroxyphenylglycol (DHPG) Sigma—D9753
(–)-Arterenol Bitartrate Salt (norepinephrine, NE) Sigma—A9512
L-3,4-Dihydroxyphenylalanine (DOPA) Sigma—D9628
(–)-Epinephrine Bitartrate Salt (EPI) Sigma—E4375
3-Hydroxytyramine Hydrochloride (dopamine, DA) Sigma—H8502
3,4-Dihydroxyphenylacetic Acid (DOPAC) Sigma—D9128
3,4-Dihydroxybenzylamine Hydrobromide (DHBA) Sigma—D7012

Equipment

Waters HPLC pump- Model 510/515
Waters 717-plus Autosampler (WISP) with refrigeration unit
ESA Coulochem II or III with conditioning and analytical cells (Dionex)
Column Temperature Controller (Cera)
Data acquisition software-Waters Empower Pro System (PC)
Water system capable of generating Type I water (e.g., Milli-Q)
Mechanical shaker or vortex mixer
Ultra-low freezer (–80 degrees C)
Vacuum pump or vacuum line
Bench top oven (dedicated for alumina storage, 90 degrees C)
Bench top balance
Refrigerator
Refrigerated centrifuge
Micro-centrifuge fitted with rotor for 1.8 ml sample tubes (e.g., Hermle)
Flammable and corrosive storage cabinets

Disposables and glassware

1.8 ml conical polypropylene sample tubes with attached caps (e.g., Eppendorf)
Adjustable volume pipetters (1000 μ l, 200 μ l, 20 μ l)

Repeater pipette
Plastic tips for above pipettes
Vials, springs and caps for WISP
250 ul disposable inserts for WISP vials
PTFE septums for WISP vials
Graduated cylinders (500ml)
Filtration device for mobile phase
Glass bottles, 1 liter
Plastic serological pipettes

Sample Collection, Storage, Handling, & Shipment

Proper sample collection, storage and shipment are critical to obtain valid results.

- Blood from patients should be collected into chilled sodium heparin tubes and immediately placed on wet ice.
- With minimal delay (preferably less than two hours), samples should be spun down in a refrigerated centrifuge at 4 degrees C for 20 minutes, at 3200 rpm.
- Plasma is then transferred into a polypropylene sample tube marked with date and patient ID and frozen immediately on dry ice.
- Storage of plasma samples must be at –80 degrees C. Catechols stored at –20 degrees C are not stable.
- Samples must be shipped on sufficient dry ice to prevent thawing during transit.

Reagents and Mobile Phases

TRIS-EDTA buffer, pH 8.6—Dissolve 20 gm of EDTA and 121 gm of TRIS base into 800 ml of HPLC grade (Type 1) water in a 1 liter glass bottle. Adjust the pH of the solution to 8.6 with concentrated HCl. QS to 1 liter. TRIS-EDTA buffer can be stored at room temperature.

20/80—This is a 20:80 (volume:volume) solution containing 20% of 0.04 M phosphoric acid and 80% of 0.2 N acetic acid. To a one liter bottle add 950 ml of HPLC grade water. Add 9.2 ml glacial acetic acid and 460 µl of phosphoric acid. QS to 1 liter. Store in the dark at room temperature.

Stock Mobile Phase—To a one liter bottle add 138 gm sodium phosphate monobasic, 500 mg EDTA, and 500 mg–700 mg of octane sulfonic acid (amount varies to optimize chromatographic separation of peaks of interest). QS to 1 liter with HPLC grade water and thoroughly dissolve. Stock mobile phase can be stored at room temperature.

Working Mobile Phase—To a one liter bottle, add 100 ml of the stock mobile phase. Add 800 ml of HPLC grade water. Add 25–35 ml of acetonitrile (amount varies to optimize chromatographic separation of peaks of interest). QS with HPLC grade water. Adjust pH of mobile phase to approximately 3.15 with phosphoric acid. Filter through a 0.22 µm type GV filter under vacuum. Transfer to a clean 1 liter bottle. The working mobile phase should be used immediately.

Standards

Stock Standards—Dilute all standards to an original concentration of 1 mg/ml in 20/80. Weigh out 10–20 mg of each standard into a tarred 20 ml scintillation vial. Use a separate spatula for each standard. Add 20/80 to result in a final concentration of 1 mg/ml, taking into account if the compound is free base or salt, as follows. For example, for NE, the amount of 20/80 to add is equal to the weight divided by 1.89; if there were 10 mg weighed out, add 10/1.89 or 5.29 ml of 20/80.

DL-3,4-Dihydroxyphenolglycol – DHPG

1 mg/ml = weight $\times$ mls to add

(–)-Arterenol Bitartrate Salt (norepinephrine, NE)

1 mg/ml = weight/1.89 $\times$ mls to add

L-3,4-Dihydroxyphenylalanine – DOPA

1 mg/ml = weight $\times$ mls to add

(–)-Epinephrine Bitartrate Salt – EPI

1 mg/ml = weight/1.82 $\times$ mls to add

3-Hydroxytyramine hydrochloride – (dopamine, DA)

1 mg/ml = weight/1.24 $\times$ mls to add

3,4-Dihydroxyphenylacetic Acid – DOPAC

1 mg/ml = weight $\times$ mls to add

3,4-Dihydroxybenzylamine hydrobromide – DHBA

1 mg/ml = weight/1.58 $\times$ mls to add

Intermediate Standards—To prepare intermediate standards of 10 μ g/ml, dilute each Stock Standard by pipetting 100 μ l of stock standard into 9.9 ml of 20/80.

Working Amine Mix—To prepare the working Amine Mix, combine 250 μ l of the Intermediate Standards for NE, EPI, and DA into a single 100 ml volumetric flask and QS with 20/80 to a final standard concentration of 250 pg of each amine/10 μ l. Aliquot 200 μ l into color-coded plastic sample tubes (e.g., yellow for Amine Mix) and store at –80 degrees C.

Working DHPG, DOPA, and DOPAC—To prepare the DHPG, DOPA, and DOPAC working standards, pipette 1 ml of the Intermediate Standards into separate 100 ml volumetric flasks and QS with 20/80 resulting in three working standards of 1 ng/10 μ l each. Aliquot 200 μ l into color-coded plastic sample tubes (e.g., DHPG green, DOPAC blue, DOPA purple) and store at –80 degrees C.

Internal Standard (DHBA)—To prepare DHBA, the internal standard, pipette 20 ml of the Intermediate Standard into a 100 ml volumetric flask and QS with 20/80. Aliquot 200 μ l into individual 2 ml screw-top cryotubes and freeze at –80 degrees C.

HPLC-EC Setup

- 1 Set up the HPLC-EC system as follows. Clean, freshly filtered mobile phase is pumped through a Waters Model 510 or 515 HPLC pump at 0.9 ml/min. The Waters pump is connected to a Waters autosampler (WISP), with the

refrigeration unit set at 4 degrees C. A reversed phase C18 HPLC column (e.g., Axxi-Chrom) is placed into a column cooler (e.g., Cera) to stabilize temperatures. The column cooler is set at 19 degrees C. The conditioning and analytical cells are placed in series after the column. Use the shortest length of tubing possible between each connection. Settings on the ESA (Dionex) Coulochem electrochemical detector should be as follows: Guard cell or conditioning cell: +0.30 V. Electrodes in the analytical cell: +0.15V and -0.35V. Response levels of the electrochemical detector are set empirically depending on the sensitivity of the system.

Assay Run Sequence

- 2 The first step in the assay is to verify that the reagents are clean. This is done by first injecting 90 µl of Type I water. The entire assay depends on the assumption that the water is perfectly clean.
- 3 The next injection is of 90 µl of 20/80, to make sure it is clean.
- 4 After verifying that the water and 20/80 are clean, 90 µl of STD MIX is injected. This injection will verify proper chromatography and the consistency in response of the system. The chromatographic peak for each analyte should be a minimum of 1 minute apart from all the other peaks, and the first peak (DHPG) should be well resolved from the solvent front (Figure 2). In the event there is confusion about the identity of the standards in the STD MIX, inject each standard separately to verify its retention time.
- 5 Once there is adequate chromatography, the samples can be pulled from the storage freezer, and the assay can be started.
- 6 For each assay run, set up tubes and plan to analyze the samples in the following sequence:
 - a mixture of directly injected standards (STD MIX)
 - extracted standards (EXT STD)
 - water blanks
 - QC samples
 - the unknowns

It is useful to prepare an assay sheet that clearly describes each sample as to ID, date, and sample amount.

Standard Mixture (STD MIX)

7. Remove from the freezer one aliquot each of the working standards for DHPG, DOPA, DOPAC, Amine Mix, and the internal standard DHBA. To the tube labeled DHBA, add 1800 µl of 20/80 and mix by inversion. To another tube labeled STD MIX, add 150 µl each of the working standards, 300 µl of the DHBA internal standard, and 450 µl of 20/80. Mix by inversion. Pipette 100 µl of STD MIX into several autosampler limited volume inserts, appropriately labeled, for direct injection into the HPLC-EC system.

Extracted Standard (EXT STD)

8. To each tube labeled EXT STD, add 90 µl of the STD MIX.

Water Blank

9. To each H₂O BLK, add 1 ml of Type 1 water.

Thaw patient and QC samples and transfer to assay sample tubes—AGAIN, great care should be taken in assuring that sample integrity was never jeopardized during collection, storage or shipment!

- 10 Centrifuge in a refrigerated centrifuge for 10 minutes at 4 degrees C, to remove particulate matter.
- 11 Label a 1.8 ml polypropylene conical sample tube with attached lid for each sample to be run.
- 12 Transfer each sample by pipetting exactly 1 ml of the centrifuged plasma into the labeled 1.8 ml polypropylene sample tube.
 - *If less than 1 ml of plasma, note how much was pipetted.*
 - *For suspected Menkes samples, run two samples, one with 1 ml and one with 20 µl, in case some of the analytes overrange.*
 - *(Standard QC plasma samples for catechols are not commercially available).*

Alumina Extraction Procedure

- 13 To each labeled 1.8 ml polypropylene sample tube with sample in it, add 5 mg of acid washed alumina. Use of a scoop from the cut off tip of a plastic limited volume insert facilitates aliquotting the correct amount of alumina.
- 14 To each tube add 500 µl of TRIS-EDTA, pH 8.6.
- 15 Add 20 µl of DHBA internal standard (water blanks and plasma samples only).
 - Cap the samples, and shake them vigorously for 30 minutes using a paint can shaker or vortex mixer.
- 16 Centrifuge in a microcentrifuge for 1 minute to spin down the alumina.
 - Aspirate the alumina supernatant and discard.
- 17 Wash the alumina with Type I water by adding about 1 ml of water, shake manually for 10–15 seconds, spin down the alumina in the microfuge, and remove the alumina supernatant, and discard the supernatant. Repeat the washing procedure.
- 18 Spin down the alumina in a microfuge. Remove excess water carefully from the surface of the alumina, using a 20 µl pipette.
- 19 Add 100 µl of 20/80 to the alumina.
 - Shake the sample in a mechanical shaker for 5 minutes.
- 20 Spin down the sample for 1 minute in a microcentrifuge.
 - Carefully remove the alumina eluate and transfer it to an autosampler vial. Do not transfer any of the alumina into the autosampler vial.
 - *If the alumina eluates cannot be run immediately, they can be wrapped and stored in a –80 degrees C freezer, indefinitely.*

- 21 Load the autosampler vials into the sample carousel and program the instrument for an Auto Run. The samples are then injected automatically sequentially onto the HPLC column.

Calculations

The Waters Empower software should be set up to calculate the results, using the EXT STD as the standard.

The equation for calculating the concentration of each analyte (using DHPG as an example) is as follows:

$$\text{Result in pg/ml} = \frac{(10/9) * (\text{DHPG peak height of the unknown}) / (\text{DHPG peak height in the EXT STD}) * (\text{DHBA peak height of the EXT STD}) / (\text{DHBA peak height of unknown}) * 1000 \text{ (250 for Amines)}}{(1000 / \text{assayed sample volume in microliters})}$$

Diagnosis of Menkes disease is based on the ratios of several of the analytes

Ratios for diagnosis of Menkes from plasma catechols are as follows:

DOPA:DHPG >6

DOPAC:DHPG >4

DA:NE > 0.10

In Menkes disease, **the DOPA:DHPG and DOPAC:DHPG ratios are always abnormally high**. DA:NE ratios also are usually high, but there can be exceptions.

Figure 3 and Figure 4 illustrate normal and abnormal chromatographs from analysis of plasma from at-risk newborn infants.

Basic Protocol 2: Copper uptake and retention in fibroblasts to diagnose Menkes Disease

Measuring of ^{64}Cu uptake and especially retention in fibroblasts is a reliable, but expensive method for postnatal diagnosis of Menkes disease. It can be used if an *ATP7A* mutation cannot be identified and the clinical and biochemical phenotype is highly suggestive. Fibroblasts from patients with Menkes disease accumulate about 2–5 times higher amounts of ^{64}Cu compared to controls. In contrast to controls, they also retain most of the ^{64}Cu after 24 hours. The test is not 100% sensitive and specific. False positive results have been observed for unknown reasons, and in cases of excess ^{64}Cu concentration in the medium, when using a specific batch of FCS, and in case of yeast infection of cells. False negative results have been observed in cases where ^{64}Cu concentration in the medium was too low, if the culture became infected with mycoplasma, or if the cultured fibroblasts grew poorly for other reasons.

Materials

Media and Chemicals

F12 (Gibco 21765-29)

FCS (Gibco 10106-169 or Sigma F7524)

Glutamax (Gibco 35050-038)

Tylosin (Sigma T3397)
Pen-Strep (Gibco 15140-122)
Amphotericin (500 IE/ml)
 ^{64}Cu (250MBq, 250 μg Cu/ml, specific activity: 1.33 TBq/g)
0.02mM EDTA (Titriplex R 3 Merck 8418) in H_2O
0.9% NaCl
10% NaOH
Hionic Flour (6013319)
Trypsin
Reagents for Lowry protein assay

Equipment

Beta scintillation-counter (Beckman LS 5000 C3)
Burker-tyrk cell counter slides
37°C cell culture incubator
Whirl-mixer
Biohit E-line Electronic Pipette e10 1.2–10 μl
Biohit E-line Electronic Pipette e120 5–120 μl
Biohit E-line Electronic Pipette e1000 50–1000 μl

Disposables and glassware

Biohit filtertips, Danddiag catalog # 783201 10 μl
Biohit filtertips, Danddiag catalog # 790101F 100/120 μl
Biohit filtertips, Danddiag catalog # 790101F 1000 μl
1.5 ml eppendorf safe-lock tubes
24 well plates

Samples

Fibroblast cultures obtained from the patients in question
Controls: at least two fibroblast cultures from known Menkes patients, and at least two fibroblast cultures from normal controls

Day 1

- 1 Heat inactivate FCS at 56°C for at least 30 min.
- 2 Mix F12 media: 800 ml F12, 200 ml heat inactivated FCS, 10 ml PEN/STREP, 15 ml Glutamax, 2.5ml tylosin and Amphotericin 2000 μl .
The cells are grown in F-12 medium seven days before ^{64}Cu is added

Day 6

- 3 Trypsinize the cells and plate in duplicate on two 24 well plates, plate A and plate B (7×10^4 cells/well, in total 28×10^4 cells). Suspend the cells in F12 media (2ml/well).

Day 7

- 4 Change the F12 medium (2ml/well).

Day 8

- 5 Add 250 μ l ^{64}Cu solution to 100 ml F12 medium, and replace the F12 medium with the medium containing ^{64}Cu (2ml/well). Seal the plates with plate-sealer.

Day 9

- 6 After 20 hours incubation, remove the medium on plate B and wash the cells with F12 (2ml/well) followed by reloading with F12 without ^{64}Cu (2ml/well). Seal the plate with plate-sealer.
- 7 Harvest the cells on plate A: Remove the medium; wash the cells (and wells) carefully with 0.02 mM EDTA. Remove the EDTA solution and replace with 0.9% NaCl (2ml/well). Remove NaCl solution and add 10% NaOH (280 μ l/well). Lyse the cells by incubation at 37°C for 30 min followed by incubation at 80°C for 10 min.
- 8 Determine the protein concentration in the NaOH cell lysate using the Lowry method (Schacterle and Pollack, 1973).
- 9 Measure accumulated ^{64}Cu by mixing 100 μ l cell lysate with 10 ml Hionic Flour. The sample is whirl-mixed, and after ½ hour in the dark placed in a beta-scintillation counter.
- 10 Because of the very short half-life ($T_{1/2}$) of ^{64}Cu = 12.8 hours (768 minutes), all calculated counts per minute (N_t) need to be recalculated to the expected number of counts per minute at time 0 (N_0), using the formula:

$$N_0 = N_t \times e^{\ln 2 \times t / (T_{1/2} \times 60)}; \text{where } t = \text{time in minutes}$$

The amount of accumulated ^{64}Cu (plate A) is reported as ng ^{64}Cu /mg protein/20 hours.

^{64}Cu -uptake results (Tønnesen and Horn 1989) from 105 patients are:

Menkes (males, n =105) 72.1 (34.3–135.1) ^{64}Cu /mg protein/20 hours

Controls (females, n =20) 18.9 (11.5–26.7) ^{64}Cu /mg protein/20 hours

Controls (males, n =21) 21.1 (9–33.3) ^{64}Cu /mg protein/20 hours

Day 10

- 11 After 24 hours in ^{64}Cu -free F12 medium, harvest plate B as described above. The protein concentration is determined and the retained ^{64}Cu determined, as described above.
- 12 The “chase” results are reported as the % of total accumulated ^{64}Cu (plate A) retained by the cells after 24 hours. Tønnesen and Horn 1989 reported data on 22 subjects:

Menkes disease (34 experiments on 16 males): 65.3 (53–4.2) % ^{64}Cu retained

Normal Controls (17 experiments on 6 males): 15.9 (13.4–23.1)% ^{64}Cu retained

Figure 5 summarizes results from affected and unaffected individuals in a scatter plot.

Basic Protocol 3: Multiplex PCR for detection of large ATP7A deletions

This protocol is used for postnatal diagnosis of Menkes disease by screening for large ATP7A deletions (Liu et al. 2002)]

Equipment

PCR Thermocycler Applied Biosystems GeneAmp PCR system 9700

Microwave oven

Electrophoresis equipment, including power supply

Materials

~300ng/ul genomic DNA (isolated from whole blood using Wizard Genomic DNA Purification Kit)

Multiplex oligonucleotide primers diluted to 10uM (5' to 3')

Primer Set A:

Exon 10 Forward: ATATATGTGAATTTTCAGCATTTTTTAA

Exon 10 Reverse: ATGTATTTCCAATGATTGGCC

Exon 20 Forward: TATTGCTCAGTTATGTTTCACGTACT

Exon 20 Reverse: GATTATCATTGACCACATAGGGC

Exon 22 Forward: ATGCTGAAGAAGTATCAAACAAAGAAA

Exon 22 Reverse: CAAGAATAAAACCTACCAAGAATGAC

Primer Set B:

Exon 1 Forward: GGGGGTGGGAAAAGAGAAGC

Exon 1 Reverse: CATGACCTTCTCACCACACTCGT

Exon 16 Forward: TTTCTGGAAAGGTGATGTGGA

Exon 16 Reverse: TAGATTTGATCCGGGGAATTC

Exon 17 Forward: AATTAAGTAGGGGTTTTTATCTTGC

Exon 17 Reverse: CAGGGTTATAAAGTTAGCCATCTG

Primer Set C:

Exon 5 Forward: GATGCAATTGAATGATCAATACTGC

Exon 5 Reverse: TGTAAGAGAACAAAAAAGATGGAGCT

Exon 12 Forward: GCATATTATCATGGTGCTTTTTAT

Exon 12 Reverse: CTCTCTGTTTATAATTTAGTTATTCATAGC

Exon 23 Forward: TCTCATTTACTTTTGTTATTTGAAACT

Exon 23 Reverse: AGTGCATGACAAGTTAACTGGC

Primer Set D:

Exon 18 Forward: GAACTTGCTTCATGGGGTTT

Exon 18 Reverse: AGAGGTCTCTCAAAAATAAATAAATTAAT

Exon 21 Forward: AAGGTAGACGTAATCTTCAACATACACAG

Exon 21 Reverse: ATGGCTTTAGTGGAAAAAGCAGTACTAGT

Primer Set E:

Exon 2 Forward: GCATGGCTGAAATTAATGAATTT

Exon 2 Reverse: CTTCAATGGCATTGACTTCAGT

Exon 7 Forward: AGTGGTAACTCATGTTTAATGGTGGGA

Exon 7 Reverse: GATTACGGGAGTGCTGATTAAA

Exon 19 Forward: TATTCCAAGTTCTTTTATTTTGTGCTG

Exon 19 Reverse: AAATTTCTTAGCATTGGAAGGCAAG

Primer Set F:

Exon 3 Forward: TGTGATAGAATTTAATTAACTGACTTTTG

Exon 3 Reverse: AAACCTCATGCTTAAGAAGAAAGCAA

Exon 4 Forward: GTGGTTATTGATTTAGAAAACAGAATG

Exon 4 Reverse: TGCCTATGAATCATTCTCATTGA

Exon 14 Forward: TACTCTGCATTGAGTATCAGAAAAGACTT

Exon 14 Reverse: CACTCCAAACCTTTAAAGATCCTGGT

Primer Set G:

Exon 8/9 Forward: GTTCTTAATGACAATACCATGGCTTAG

Exon 8/9 Reverse: GCCTGCCAAATTTTGCTTATT

Exon 11 Forward: AGACCTGAACTTTTCTTCCTTAGC

Exon 11 Reverse: TAAAAAAGAGAAGAGGGAAGAGTG

Exon 15 Forward: TTGTACAGCTCTAAATCAATAACCA

Exon 15 Reverse: GTGCTATTGATAGTTTGGTGGTAC

Primer Set H:

Exon 6 Forward: TTTCATACTTGATAATACTAAGAAGTAAT

Exon 6 Reverse: CAACTCTTCTCTGAACCTTACCAAG

Exon 13 Forward: AACAAATAATGATATGTTTCATGATAGTG

Exon 13 Reverse: GTGAAGATTTATACTGAGAAGGCTTTA

10 mM dNTP mix (Invitrogen)

5 U/ul Taq DNA Polymerase High Fidelity (Invitrogen)

Buffer D (Invitrogen)

PCR Water
 NuSieve agarose (Lonza)
 1X TBE Buffer
 10 mg/ml Ethidium Bromide Solution (Invitrogen)
 6X gel loading buffer
 GeneRuler DNA Ladder Mix (Fermentas)

Protocol

Label eight PCR tubes A through H and dilute primers to 10uM. Set up the PCR amplification reactions for a final volume of 50ul. Include a normal male control DNA sample.

1ul template genomic DNA
 5ul primer (A–H) (10uM each)
 10ul 5X Buffer D
 2.5ul 10mM dNTP mixture
 0.3ul Platinum Taq High Fidelity DNA Polymerase
 31.2ul PCR water

2 Perform PCR using the following protocol:

Initial step:	5min 95°C (denaturation)
30 cycles:	30s 95°C (denaturation)
	45s 55°C (annealing)
	2min 75°C (extension)
Final step: Store at 4°C	7min 72°C

3 Prepare a 2% agarose gel solution by slowly adding 1g of agarose to 50ml of 1X TBE. Mix thoroughly and heat in a microwave twice for 30 second intervals, swirling the mixture after each heating. Let solution cool slightly and add 2ul of ethidium bromide. Swirl to completely mix the solution. Immediately cast in appropriate size gel tray, e.g., 15 × 10cm. Insert combs that are between 3.5mm and 4.5mm wide in order to load 20ul in each well.

4 When the gel has polymerized (approximately 30 minutes), place gel in 1X TBE buffer and remove combs. Load each well with a mixture of 15ul PCR product (from step 2) with 3ul of 6X gel loading buffer. Load ~15ul of ladder in separate lane.

5 Run the gel at 85V for ~30 to 45 minutes. Visualize and photograph the gel on an ultraviolet light box using a gel photography system

6 Expected fragment size:

Reaction A:

Exon 10: 376 base pairs

Exon 20: 332 base pairs

Exon 22: 287 base pairs

Reaction B:

Exon 1: 262 base pairs

Exon 16: 306 base pairs

Exon 17: 371 base pairs

Reaction C:

Exon 5: 331 base pairs

Exon 12: 291 base pairs

Exon 23: 369 base pairs

Reaction D:

Exon 18: 295 base pairs

Exon 21: 258 base pairs

Reaction E:

Exon 2: 219 base pairs

Exon 7: 290 base pairs

Exon 19: 259 base pairs

Reaction F:

Exon 3: 624 base pairs

Exon 4: 904 base pairs

Exon: 14: 323 base pairs

Reaction G:

Exon 8/9: 649 base pairs

Exon 11: 182 base pairs

Exon 15: 294 base pairs

Reaction H:

Exon 6: 327 base pairs

Exon 13: 291 base pairs

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Interpretation: DNA from a normal male should be run as a control for the multiplex assay. Deletions are easily evident from visual comparison of the subject's gel pattern in comparison to the normal control. Deletions are predictive of Menkes disease, rather than one of the other ATP7A variants,

Basic Protocol 4: PCR and Sequencing for Detection of Small ATP7A Alterations

When large gene deletions have been excluded, a search for smaller genetic alterations may proceed. Although heteroduplex analysis has been used, direct sequence analysis is

somewhat simpler. Missense mutations and single base insertions or deletions can be readily detected.

Materials

Chemicals

Hot Start Taq (Qiagen Catalog # 203205)

AmpliTaq Gold polymerase (Applied Biosystems, Catalog # N808-0248)

10 x PCR-buffer containing 15mM MgCl₂ (Catalogue # 4396)

5 x Q-solution

10 x PCR-buffer containing 15mM MgCl₂ (Catalogue #1005479)

dNTP (2.5mM of each nucleotide)

Primer mix for PCR amplification (forward and reverse, 10μM each; Table 1)

H₂O (milli-Q)

PCR-tubes, 96-well PCR plates

Multiscreen (Milipore, to be used for purification prior to sequencing)

Thermowell Sealing Tape

TE-buffer

Agarose

Ethidium Bromide (10 mg/ml)

Cresol red

TBE-buffer

Loading buffer

DNA size standard ladder

Oligonucleotide primers for sequencing:

accactgcttactggttacc (pS) for all forward reactions

gaggggcaaacaacagatggc (pC) for all reverse reactions

BigDye Terminator v3.1 Cycle Sequencing Kit, 4337035 rev A

BigDye Terminator Sequencing buffer

Sequencing Wash buffer

Injection solution buffer

0.125 M EDTA,

96% ethanol

70% ethanol

Salsa MLPA P104, Menkes disease (MRC-Holland, Amsterdam, The Netherlands)

Formamide

Rox500 standard

Equipment

PCR Thermocycler Applied Biosystems GeneAmp PCR system 9700
 Microwave oven
 Electrophoresis equipment, including power supply
 Vacuum-pump
 Centrifuge (Heraus Labofuge 400R)
 Minishaker (for 96 well plates)
 ABI 3130XL Genetic Analyzer (Applied Biosystems)
 Secscape/Gene Marker v.1.95 (Applied Biosystems)
 96 well plate centrifuge (Laboratory Medical Centrifuge, Biosan)
 Biohit E-line Electronic Pipette e10 1.2–10 µl
 Biohit E-line Electronic Pipette e120 5–120 µl
 Biohit E-line Electronic Pipette e1000 50–1000 µl

Disposables and glassware

Biohit filtertips, Danddiag catalog # 783201 10 µl
 Biohit filtertips, Danddiag catalog # 790101F 100/120 µl
 Biohit filtertips, Danddiag catalog # 790101F 1000 µl
 1.5 ml Eppendorf safe-lock tubes
 MicroAmp 96-well tray (Applied Biosystem part # 403081/VWR 82006-636)
 PCR tubes
 Vials 50 ml for agarose,
 Glass bottles for agarose

Samples

Genomic DNA extracted from blood or fibroblasts. For the MLPA test, EDTA blood gives the best result.

All forward primers are tagged with the sequence: acccactgcttactggcttacc (pS) and all reverse primers with the sequence: gaggggcaaacacagatggc (pC) respectively. Because exon 3 and exon 4 is very large they are amplified in overlapping Fragments 3a, 3b and 4a, 4b, 4c respectively.

A) PCR amplification and sequencing of the 23 exons

- 1 Set up the following PCR amplification reactions for the 23 exons. A blank sample (no DNA) should be included as a control for DNA contamination.

Exon 1:	Exon 2–23:
14.4 µl H ₂ O	20.3 µl H ₂ O
6 µl 5x Q-solution	3 µl 10 × PCR Buffer (catalog #4396)

3 μ l 10 $\times$ PCR Buffer (catalog #1005479)	0.5 μ l dNTP
0.5 μ l dNTP	0.2 μ l Taq Gold
0.1 μ l Hot Star Taq	1 μ l Primer (mix of forward and reverse primers)
1 μ l Primer (mix of forward and reverse primers)	5 μ l DNA (20 ng/ μ l)
5 μ l DNA (20 ng/ μ l)	

2 Perform PCR using the following amplification protocols:

Exon 1	95°C (15 min) 95°C (1 min), 60°C (−0.5°C/cycle) (1 min), 72°C (1 min), 14 cycles 95°C (1 min), 53°C (1 min), 72°C (1 min), 26 cycles 72°C (7 min) 4°C
Exon 2, 4 (a,b,c), 6, 7, 9–11, 13, 15–23	95°C (7 min) 95°C (30 sec), 63°C (−0.5°C/cycle) (30 sec), 72°C (1 min), 14 cycles 95°C (30 sec), 56°C (30 sec), 72°C (1 min), 26 cycles 72°C (7 min) 4°C
Exon 3 (a,b), 5, 8, 12, 14	95°C (7 min) 95°C (30 sec), 70°C (−0.5°C/cycle) (30 sec), 72°C (1 min), 14 cycles 95°C (30 sec), 63°C (30 sec), 72°C (1 min), 26 cycles 72°C (7 min) 4°C

- 3** Test the production of PCR fragments by gel-electrophoresis. Prepare a 2% gel solution. For 500 ml weight out 10g of agarose into a 1000 mL conical flask. Add 500mL of 1 $\times$ TBE, swirl to mix. Microwave for about $2 \times 2 \times 1$ minutes to dissolve the agarose. The agarose solution can boil over very easily so keep checking it. Leave it to cool on the bench for 5 minutes down to about 60°C (keep it in oven at 60°C). Take 30 ml from the agarose solution, into a 50 ml plastic tube. Add 1 μ l of ethidium bromide, mix and cast in a gel tray for electrophoresis, insert combs for 10 μ l wells. When the gel has polymerized, add 1 $\times$ TBE to cover the gel and remove the combs. Load a mixture of 5 μ l PCR product and 4 μ l cresol red in each well. Run the gel at 80 V for 20–40 min and analyze in a gel reader (using UV light, it is possible to see the PCR products because Ethidium bromide is bound to the DNA). Print a photo.

Nota bene, ethidium bromide is mutagenic.

- 4** Remove excess dNTP and primers from the successful PCR reactions with the Millipore DNA Purification Kit, according to the manufacturer's instructions.
- 5** Set up two sequencing reactions for each PCR product using the pS or pC primer respectively by mixing:

For all exons:

4 µl 2.5x Big Dye Terminator Ready Reaction mix
 0.3µl primer pS/pC
 5 µl purified PCR product (2 ng/µl)
 0.7µl H₂O

Perform PCR with the following protocols:

Exon 1:	98°C (1 min)
	98°C (10 sec), 50°C (5 sec), 60°C (4°C)
	4°C
Exon 2–23:	96°C (1 min)
	96°C (10 sec), 50°C (5 sec), 60°C (4°C)
	4°C

- 6 Remove excess Big Dye Terminator Reaction mix with the Multiscreen according to the manufacturer's instructions. PCR products dissolved in injection solution next are loaded on the ABI 3130XL Genetic Analyzer.
- 7 Analyze the data with SeqScape to identify mutations. For comparison, use the Genbank ATP7A sequence NM_000052 sequence where A in the ATG start codon is numbered as c.1 and the TG codon as p.1.

Alternate Protocol 1: Multiplex Ligation-dependent Probe Amplification (MLPA) to Detect ATP7A Deletions and Duplications

CPHG Unit 7.14 describes theory behind MLPA. It is used to test for exon deletions or duplications using the Salsa MLPA kit P104, Menkes disease, according to the manufacturer's instructions (MRC-Holland). The kit contains probes for all 23 exons of the ATP7A gene, and some control probes as well. Details on probe sequences can be found at the company's website (<http://www.mrc-holland.com>). All runs should include DNA from two unrelated unaffected males. The best results are obtained if the genomic DNA is extracted from EDTA blood.

In contrast to Basic Protocol 3, MLPA detects exon duplications (approximately 4 % of ATP7A mutations appear to be duplications of one or several exons) as well as deletions. Since the method is quantitative, detection of ATP7A gene deletions and duplications in female carriers is also possible.

Day 1

DNA denaturing

- 1 Place 5 µl DNA (50 ng/µl) in a PCR tube/9 well in a thermo-cycler,
 98 °C (5 min)
 25 °C (pause)

Hybridization

- 2 Add 1,5 µl Probe mix and 1,5 µl SALSA MLPA buffer. Mix by centrifuge.

95 °C (1 min)
60 °C (for at least 16 hours)

Day 2

Ligation

3 Program the temperature in the thermocycler to 54°C.

Add 25 µl H₂O, 3 µl Ligase-buffer A, 3 µl Ligase-buffer B and 1 µl Ligase and mix by centrifuge. Add to the 8µl DNA mix.

54°C (15 min),

98°C (5 min)

4°C (pause)

PCR-reaction

4 Mix 26 µl H₂O and 4 µl SALSA PCR-buffer with 10µl ligation product in a new PCR tubes/96 plate well at room temperature in a thermocycler.

5 Polymerase mix: 5.5 µl H₂O + 2 µl SALSA FAM PCR-primer + 2 µl SALSA enzyme dilution-buffer + 0.5 µl polymerase. Mix by centrifugation and leave on ice.

6 Heat the thermal-cycler to 60°C and add the polymerase mix to the PCR tubes/96well plate. Perform PCR using the program:

95°C (30 sec), 60°C (30 sec), 72°C (1 min); 35 cycles

72°C (20 min)

4°C (pause).

7 Mix 14.63 µl formamide and 0.37 µl ROX500 standard with 1.2µl PCR product and run on an ABI3130XL Genetic Analyzer (using filter D in 11 sec)

8 Analyze the data with Gene Marker (regression analysis and population method) to obtain peak areas to perform exon quantitation. Figure 6 shows the result from a patient with deletion of ATP7A exons 5–16 (panel B).

For further information see the homepage www.mrc-holland.com

4. Chorionic Villus Specimen Copper Level—If the family specific mutation is not identified, prenatal diagnosis by measuring the copper level in chorionic villus specimen (CVS) is possible (Heydorn K et al., 1999). For the CVS copper level test, the Kennedy Center sends a copper-free vial, a CVS-washing solution and filter paper, in advance. The instruments used for obtaining the CVS should be stainless steel, in excellent condition, and not contaminated by copper. The amount of copper in the CVS today is measured using Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

The CVS copper test is not 100% sensitive or specific for prenatal diagnosis of Menkes disease. In four of 240 total cases, known false positive results occurred, presumably due to copper contamination of the CVS (Heydorn K et al., 1999). The risk for false negative results is not considered high (no known examples in our experience).

Basic Protocol 5. PCR and Sequencing for *ATP7B* mutation analysis

The molecular diagnosis of Wilson disease (MIM 277900) is performed by identification of two disease-causing mutations in the *ATP7B* gene (MIM 606882). Wilson disease is autosomal recessive and most patients are compound heterozygotes. Screening for base-pair substitutions and small base pair deletions, duplications or insertions are performed by sequencing the 21 exons including 20 base pair flanking sequences. If no mutations are identified by sequencing, screening for exon deletions or duplications can be performed using Multiplex Ligation-dependent Amplification (MLPA; see Alternate Protocol 2) (Schouten et al., 2002). In contrast to *ATP7A*, only a few examples of exon deletions have been identified in the *ATP7B* gene and no exon duplications have been identified in the *ATP7B* gene, to our knowledge.

The Genbank sequence NM_000053 is used as reference sequence where A in the ATG start codon is numbered as c.1 and the ATG codon as p.1. For identified mutations see the Wilson Disease Mutation Database www.wilsondisease.med.ualberta.ca/database.asp and <http://www.hgmd.cf.ac.uk/ac/index.php>.

Materials

As described for *ATP7A*, except that AmpliTaq Gold polymerase (Applied Biosystems, catalog # N808-0248) and 10x PCR-buffer containing 15mM MgCl₂ (catalog # 4396) is used for all PCR amplifications (no need for Hot start Taq (Qiagen Catalog # 203205), 5x Q-solution, nor 10x PCR-buffer containing 15mM MgCl₂ (catalog # 1005479). For MLPA, the SALSA MLPA kit P098, Wilson disease (MRC-Holland) is used.

ATP7B Primers for exon PCR-amplification:

Exon	Primer (L:left, R:right)	Sequence Lower letters:universal tags, upper letters:gene specific	Expected size fragment (base pair)
1	WNDex1L	accactgttactggttaccGCAACTTTGAATCATCCGTG	381
	WNDex1R	gaggggcaacaacagatggcAACGCGGGGAGGAAATC	
2	WNDex2aL	accactgttactggttaccCACCAGTGGCATTGTTTCC	508
	WNDex2aR	gaggggcaacaacagatggcAAGGACCTTGAGGGCCAG	
	WNDex2bL	accactgttactggttaccTGTGAAATATGTGCCATCGG	463
	WNDex2bR	gaggggcaacaacagatggcGCAGAAGATAAAGGTCTCTTTGG	
	WNDex2cL	accactgttactggttaccAGCTGCCATCAAGAGCAAAG	459
	WNDex2cR	gaggggcaacaacagatggcGGGAATGAGAACTGGAAGACC	
	WNDex2dL	accactgttactggttaccTCTGCAGAGGGCTATCGAG	514
	WNDex2dR	gaggggcaacaacagatggcCCACTGTTGACATGGGAGG	
3	WNDex3L	accactgttactggttaccCGGGACAATGAACCCTCAC	512
	WNDex3R	gaggggcaacaacagatggcTGCTACCTGGTTATCAGGGC	
4	WNDex4L	accactgttactggttaccTTTGAAACTGCTGTTCTAGAGG	435
	WNDex4R	gaggggcaacaacagatggcACCAGACACGTCCAAGATGG	
5	WNDex5L	accactgttactggttaccGGAAAGGCTCTTGGCTGC	435
	WNDex5R	gaggggcaacaacagatggcTTCCATGGGAAAAGTTGAAG	

Exon	Primer (L:left, R:right)	Sequence Lower letters:universal tags, upper letters:gene specific	Expected size fragment (base pair)
6	WNDex6L	accactgttactggttaccCCCACAAAGTCTACTGAGGC	350
	WNDex6R	gaggggcaacaacagatggcAAGGCACCATGGGAAGAC	
7	WNDex7L	accactgttactggttaccTCCCAGACATGTGACAAAGG	443
	WNDex7R	gaggggcaacaacagatggcAAAGTGCCATTAAACCAAGC	
8	WNDex8L	accactgttactggttaccATAAACGCCCATCACAGAGG	567
	WNDex8R	gaggggcaacaacagatggcGCACCTTAATTATATGGAGGTTCC	
9	WNDex9L	accactgttactggttaccGCTGTTTCTCTCGACACAG	359
	WNDex9R	gaggggcaacaacagatggcTCTTGCCACACTACAAG	
10	WNDex10L	accactgttactggttaccAACAGATCAAATGAATACTGTTGC	444
	WNDex10R	gaggggcaacaacagatggcTGCTTCCGGGTTTCTTAGTG	
11	WNDex11L	accactgttactggttaccATGGCTTGTTCATGTTCCC	453
	WNDex11R	gaggggcaacaacagatggcCAAGCATGGATAAAGATTGGG	
12	WNDex12L	accactgttactggttaccTGTGAAGAGTTCTGGGAAATCAG	487
	WNDex12R	gaggggcaacaacagatggcGGCTTAGATTTTGCTGTCAATAAG	
13	WNDex13L	accactgttactggttaccGGATAGCTGGGATGTGGAGA	521
	WNDex13R	gaggggcaacaacagatggcTGAGTGGCTCTCAGGCTTTT	
14	WNDex14L	accactgttactggttaccTGCAGGTGTCTTGTTCCTG	456
	WNDex14R	gaggggcaacaacagatggcTGGTTTCCAGACCACACAG	
15	WNDex15L	accactgttactggttaccCTCTCTTGCCACCTTCACCC	432
	WNDex15R	gaggggcaacaacagatggcCCTTAGCCATGAACCGTCTG	
16	WNDex16L	accactgttactggttaccTGCTGTCAAGAGGTGCTTAC	456
	WNDex16R	gaggggcaacaacagatggcCAGGCCTGAAATTAAGAGAGG	
17	WNDex17L	accactgttactggttaccGACTTTTGTGTACATCCGTAAATG	529
	WNDex17R	gaggggcaacaacagatggcGCAACTACATGGCCACAG	
18	WNDex18L	accactgttactggttaccCAACATCACTGACTGGACCC	477
	WNDex18R	gaggggcaacaacagatggcTGAATGCTAGCCACCACATC	
19	WNDex19L	accactgttactggttaccAGCCGACGTCGTCCTTATC	378
	WNDex19R	gaggggcaacaacagatggcCTAAAACGCCTCTAGCCAGC	
20	WNDex20L	accactgttactggttaccCACGCCAGGTGGAGACC	375
	WNDex20R	gaggggcaacaacagatggcTTGTCCCAGGTGAATGAATG	
21	WNDex21L	accactgttactggttaccAAAAGCCTTGTTTCTAGAAT	476
	WNDex21R	gaggggcaacaacagatggcAGTCCCAAAGCTGGAGG	

PCR amplification and sequencing of the 21 ATP7B exons

- 1 Perform PCR amplification and sequencing as described for the ATP7A gene with the following modifications:

Set up the following PCR amplification reactions for the 21 exons:

Exon 1–21:

20,3 µl H₂O
 3 µl 10 x PCR Buffer (Catalog # 4396)
 0.5µl dNTP
 0.2µl Taq Gold
 1µl Primer (a mix of forward and reverse primers)
 5µl DNA (20 ng/µl)

- 2 Perform PCR using the following amplification protocols:

Exon 1–21	95°C (7 min)
	95°C (30 sec), 63°C (–0.5°C/cycle) (30 sec), 72°C (1 min), 14 cycles
	95°C (30 sec), 56°C (30 sec), 72°C (1 min), 26 cycles
	72°C (7 min)
	4°C

- 3 Set up two sequencing reactions for each PCR product using the pS or pC primer respectively by mixing:

For all exons:
 4 µl 2.5x Big Dye Terminator Ready Reaction mix
 0.3µl primer pS/pC
 5 µl purified PCR product (2 ng/µl)
 0.7µl H₂O

- 4 Perform PCR with the following protocols:

Exon 1–21:	96°C (1 min)
	96°C (10 sec), 50°C (5 sec), 60°C (4°C)
	4°C

- 5 Test the production of PCR fragments by gel-electrophoresis. Prepare a 2% gel solution. For 500 ml weight out 10g of agarose into a 1000 mL conical flask. Add 500mL of 1 x TBE, swirl to mix. Microwave for about 2 × 2 × 1 minutes to dissolve the agarose. The agarose solution can boil over very easily so keep checking it. Leave it to cool on the bench for 5 minutes down to about 60°C (keep it in oven at 60°C). Take 30 ml from the agarose solution, into a 50 ml plastic tube. Add 1µl of ethidium bromide, mix and cast in a gel tray for electrophoresis, insert combs for 10µl wells. When the gel has polymerized, add 1 x TBE to cover the gel and remove the combs. Load a mixture of 5µl PCR product and 4µl cresol red in each well. Run the gel at 80 V for 20–40 min and analyze in a gel reader (using UV light, it is possible to see the PCR products because Ethidium bromide is bound to the DNA). Print a photo.

Nota bene, ethidium bromide is mutagenic.

- 6 Remove excess dNTP and primers from the successful PCR reactions with the Millipore DNA Purification Kit, according to the manufacturer's instructions.
- 7 DNA Sequence analysis:
DNA sequences derived from individual PCR fragments are compared to the normal expected sequences via the basic local alignment search tool (BLAST), National Center for Biotechnology Information, U.S. National Library of Medicine.

Alternate Protocol 2: MLPA test for ATP7B

To test for exon deletions or duplications, use the MLPA kit Salsa P098 according to the manufacturer's instructions (MRC-Holland). The kit contains probes for all 21 exons of the ATP7B gene and appropriate control probes. Details on probe sequences can be found at the company's website (<http://www.mrc-holland.com>). All runs included at least DNA from 2 unrelated unaffected controls. The best results are obtained if the genomic DNA is extracted from EDTA blood.

For the rest of this procedure, please see ATP7A

Basic Protocol 6: Ceruloplasmin (CP) Gene Mutation Analysis

Ceruloplasmin is part of a panel of genes that can be screened for alterations that may affect normal copper metabolism in patients. The analysis is performed by PCR amplification of coding sequences of an individual's DNA, followed by purification and direct sequencing of the PCR fragments.

Materials

~300ng/ul genomic DNA (isolated from whole blood using Wizard Genomic DNA Purification Kit)

Ceruloplasmin oligonucleotide primers (5' to 3'):

Exon 1 Forward: CTGCCCTCCTGGAATTTACACAATG

Exon 1 Reverse: TGTCTTGGGTTTCAAGCCCACA

Exon 2 Forward: CAGGCAAACATTCAATGTGATGTGC

Exon 2 Reverse: GGAAATTCAGCTACATGAGCTG

Exon 3 Forward: ACTTTGCACCTTCATTGCATGTTGC

Exon 3 Reverse: ACAAAGTGGCCTGCCCCTGT

Exon 4 Forward: AACAGGAGTGACTGGTCCAGACTT

Exon 4 Reverse: AAGGACCACAGACTAGACACACAGT

Exon 5 Forward: AGGGGGCAAGAATACCAGCATGT

Exon 5 Reverse: CCCAGTAACCACCTTTTTCAGCTGA

Exon 6 Forward: CCCGAGCAGTGTTTACAGGGC

Exon 6 Reverse: CCTTTGTGCGGGGAGAGCAT

Exon 7 Forward: GTCAAGGGGTTGAAGGGGCCCT

Exon 7 Reverse: CCCATGGGAAGAGTAAACCAGCCA

Exon 8 Forward: GGGACAAATGACACACCTCCAGCC
Exon 8 Reverse: CGGTTTCCTTGGGAGTTCCTGCC
Exon 9 Forward: AGGTGCTAGGGACATTCCCGGAA
Exon 9 Reverse: ATGTTGGGGACCCCTGTGTAAATG
Exon 10 Forward: TCCCTGATTTCCAGCACAGACCCA
Exon 10 Reverse: TGAGCCTGTCATTTTTGAGCCAAAA
Exon 11 Forward: AGCCAGCGGCTGCTCTGAAATG
Exon 11 Reverse: AGGGCACTTCAGAGGCTTGGGGA
Exon 12 Forward: GGAGAGCCCCAGAGGGCACTAG
Exon 12 Reverse: GCACATGAATGTGAGAAGACACTCC
Exon 13 Forward: GCCAGGACTCAGATGGCTGCAC
Exon 13 Reverse: TTGACGGTTACTGCAGGTAGCATCA
Exon 14 Forward: ACCACAAACCCATGGGGGAGAGAA
Exon 14 Reverse: AGACACCTCCTTGCATCCCCTCT
Exon 15 Forward: TGACATTTCCAGTTCCTGGGTCCT
Exon 15 Reverse: GTCTTGTGTGTCATAGGTAGCAGCA
Exon 16 Forward: TTCCTGAGGCCCTGGGTGCA
Exon 16 Reverse: ACCTGACATACAAAGTGAGGCAGAA
Exon 17 Forward: ACCACTTCTGCCTCACTTTGTATGT
Exon 17 Reverse: TGTGTCAATTGAAATTGCTGTGCCT
Exon 18 Forward: CACACCCACTTGATGTGGATACCA
Exon 18 Reverse: AGGGCAGCAGATTCACAAATGCT
Exon 19 Forward: CGG ATC ATG TAC TGC ATT GTT GCC
Exon 19 Reverse:GGTGTGATGTCAACAGCATGTT

10 mM dNTP mix (Invitrogen)

5 U/ul Taq DNA Polymerase High Fidelity (Invitrogen)

10X High Fidelity PCR Buffer (Invitrogen)

50 mM MgSO₄

PCR water

NuSieve agarose (Lonza)

1X TBE Buffer

10 mg/ml Ethidium Bromide Solution (Invitrogen)

6X gel loading buffer

GeneRuler DNA Ladder Mix (Fermentas)

Protocol

1 Set up PCR amplification reactions in PCR tubes for final volume of 50ul:

1ul template genomic DNA
 2 ul CP forward and reverse primers (10uM each)
 5ul 10X High Fidelity PCR Buffer
 1ul 10mM dNTP mixture
 2ul 50mM MgSO₄
 .2ul Platinum Taq High Fidelity DNA Polymerase
 38.8ul PCR water

2 Perform PCR using the following protocol:

Initial step:	3min 94°C (denaturation)
35 cycles:	30s 94°C (denaturation)
	30s 57°C (annealing)
	1.5min 72°C (extension)
Final step: Store at 4°C	7min 72°C

3 Prepare a 2% agarose gel solution by slowly adding 1g of agarose to 50ml of 1X TBE. Mix thoroughly and heat in a microwave twice for 30 second intervals, swirling the mixture after each heating. Let solution cool slightly and add 2ul of ethidium bromide. Swirl to completely mix the solution. Immediately cast in 10×15cm gel tray. Insert combs in order to load 20ul in each well.

4 When the gel has polymerized (approximately 30 minutes), place gel in 1X TBE buffer and remove combs. Load each well with a mixture of 15ul PCR product (from step 2) with 3ul of 6X gel loading buffer. Load ~15ul of ladder in separate lane.

5 Run the gel at 85V for ~30 to 45 minutes

Expected fragment size:

Exon 1: 580 base pairs
 Exon 2: 400 base pairs
 Exon 3: 304 base pairs
 Exon 4: 425 base pairs
 Exon 5: 436 base pairs
 Exon 6: 303 base pairs
 Exon 7: 459 base pairs
 Exon 8: 598 base pairs
 Exon 9: 887 base pairs
 Exon 10: 946 base pairs
 Exon 11: 521 base pairs

Exon 12: 537 base pairs

Exon 13: 427 base pairs

Exon 14: 373 base pairs

Exon 15: 397 base pairs

Exon 16: 433 base pairs

Exon 17: 933 base pairs

Exon 18: 489 base pairs

Exon 19: 943 base pairs

7 PCR fragment purification:

All the individual PCR fragments are excised, purified using the QIAquick PCR Purification Kit Protocol (Qiagen, Valencia, CA USA), and submitted for automated DNA sequencing.

8 DNA Sequence analysis:

DNA sequences derived from individual PCR fragments are compared to the normal expected sequences via the basic local alignment search tool (BLAST), National Center for Biotechnology Information, U.S. National Library of Medicine.

Basic Protocol 7: CCS Gene Mutation Analysis

CCS, the ubiquitously expressed copper chaperone for superoxide dismutase 1, is responsible for the delivery of copper to SOD1. The analysis is performed by PCR amplification of the eight exons including intron-exon boundaries of CCS (Gene bank accession number: NG_008733) of an individual's DNA, followed by purification and direct sequencing of the PCR fragments.

Materials

~250ng/ul genomic DNA (isolated from whole blood)

CCS oligonucleotide primers (5' to 3'):

Exon 1 Forward: CCA GGA ACA GAA AGC CG

Exon 1 Reverse: CCC AAC CCA AGT GCC C

Exon 2 Forward: GGC TTG TTC CCA GAC CCT TG

Exon 2 Reverse: GCC TTT CAC TCT CTG GGG TTC

Exon 3–4 Forward: GAG GCT TAC TCA GGG TTA CAC AG

Exon 3–4 Reverse: GGT TGT GCC TGT GTG TAT GTG C

Exon 5 Forward: GTC TGA TTC TTC TCG GTG CCC

Exon 5 Reverse: CCA CCA AAA AGG AAT CAC ACT G

Exon 6–7 Forward: CAC TTT CCA CTT CCA GAC AGG GC

Exon 6–7 Reverse: CAG AAG AAT GTG TGG TCA GGG GTG GG

Exon 7–8 Forward: CAT CAT CTG AAG CTG TCG TCT CCC

Exon 7–8 Reverse: GGC TCC AAT GCT AAG GGG TG

Exon 3seq Reverse: CCT CAA AGC CAC TCC AAC CC

Exon 4seq Forward: GGG TTG GAG TGG CTT TGA GG

3.75 mM dNTP mix (Peqlab)

5 U/ul Taq DNA Polymerase (QIAGEN)

10x PCR Buffer (QIAGEN)

PCR water

Agarose (Bioline)

1X TAE Buffer

0.5% [v/v] GelRed Nucleic Acid Stain (Biotrend)

1x gel loading buffer

GeneRuler DNA Ladder Mix (Fermentas)

Protocol

1 Set up PCR amplification reactions in PCR tubes for final volume of 50ul:

1 ul template genomic DNA

2 ul forward and reverse primers (10 pmol each)

5 ul 10x PCR Buffer

2.6 ul dNTP mixture (3.75 mM)

0.5 ul Taq DNA Polymerase

36.9 ul PCR water

2 Perform PCR using the following protocol:

Initial step:	5min 95°C (denaturation)
35 cycles:	30s 95°C (denaturation)
	30s x°C (annealing)
	y s 72°C (extension)
Final step:	Store at 4°C 7min 72°C

Exon	Annealing (x) [°C]	Extension (y) [sec]
1	63	35
2/3–4	61	40
5	58	35
6–7	65	45
7–8	63	45

3 Prepare a 2% agarose gel solution by using 1X TAE buffer. Mix thoroughly and heat in a microwave, swirling the mixture in between. Let solution cool slightly and add 1–2 µl of EtBr or 0.5% [v/v] of GelRed Nucleic Acid Stain. Swirl to completely mix the solution. Immediately cast in appropriate size gel tray and insert comb.

4 When the gel has polymerized (approximately 30 minutes), place gel in 1x TAE buffer and remove combs. Load each well with a mixture of 1ul PCR product (from step 2) with 5ul of 1x gel loading buffer. Load 1 ul of DNA ladder in separate lane.

5 Run the gel at 85V for ~30 to 45 minutes.

6 Expected fragment size:

Exon 1: 369 base pairs

Exon 2: 303 base pairs

Exon 3–4: 778 base pairs

Exon 5: 330 base pairs

Exon 6–7: 980 base pairs

Exon 7–8: 949 base pairs

7 PCR fragment purification:

The individual PCR fragments are purified using the High Pure PCR Product Purification Kit (Roche, Mannheim, Germany) and analyzed by direct sequencing on an ABI 377 semi-automated sequencer using the DNA Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions.

8 DNA Sequence analysis:

DNA sequences derived from individual PCR fragments are compared to the normal expected sequences from the National Center for Biotechnology Information (U.S. National Library of Medicine) using the DNASTAR 5.07 software.

Basic Protocol 8: CTR1 Gene Mutation Analysis

Although mutations in CTR1 have not been reported, it is a crucial membrane protein responsible for transporting copper into cells. Evaluating this gene is reasonable if mutations are absent in the other suspected genes and a patient presents with strong clinical evidence of a copper transport disorder. The analysis is performed by PCR amplification of coding sequences of an individual's DNA, followed by purification and direct sequencing of the PCR fragments.

Materials

~300ng/ul genomic DNA (isolated from whole blood using Wizard Genomic DNA Purification Kit)

CTR1 oligonucleotide primers at 10uM (5' to 3'):

Exon 1 Forward: TTGCAGGGATTGGTCGGTAATGC

Exon 1 Reverse: ACAGGGGTGCGAAAGAGGG

Exon 2 Forward: CTGATCAGTAGTGGGATCATGCC

Exon 2 Reverse: TACCCAAGTGGGTGGGAAAG

Exon 3 Forward: GGTGCCTGTGGTGGTAACGCT

Exon 3 Reverse: AGAAAGAGGTGCTGGACTTC

Exon 4 Forward: CAGCACATTGGCTAATGATT

Exon 4 Reverse: AAGAAGAGGGCTGCTGCTGATTC

Exon 5 Forward: TGCAGAGAATCTAGGAGTCC

Exon 5 Reverse: GCTCTAGAACTGCAATCGATAAGGCCACGA

10 mM dNTP mix (Invitrogen)

5 U/ul Taq DNA Polymerase High Fidelity (Invitrogen)

10X High Fidelity PCR Buffer (Invitrogen)

50 mM MgSO₄

PCR water

NuSieve agarose (Lonza)

1X TBE Buffer

10 mg/ml Ethidium Bromide Solution (Invitrogen)

6X gel loading buffer

GeneRuler DNA Ladder Mix (Fermentas)

Protocol

- 1 Set up PCR amplification reactions in PCR tubes for final volume of 50ul:
 - 1ul template genomic DNA
 - 2 ul CTR1 forward and reverse primers (10uM each)
 - 5ul 10X High Fidelity PCR Buffer
 - 1ul 10mM dNTP mixture

2ul 50mM MgSO₄

.2ul Platinum Taq High Fidelity DNA Polymerase

38.8ul PCR water

2 Perform PCR using the following protocol:

Initial step:	5min 95°C (denaturation)
30 cycles:	30s 95°C (denaturation)
	45s 62°C (annealing)
	1.5min 72°C (extension)
Final step: Store at 4°C	7min 72°C

3 Prepare a 2% agarose gel solution by slowly adding 1g of agarose to 50ml of 1X TBE. Mix thoroughly and heat in a microwave twice for 30 second intervals, swirling the mixture after each heating. Let solution cool slightly and add 2ul of ethidium bromide. Swirl to completely mix the solution. Immediately cast in appropriate size gel tray. Insert combs in order to load 20ul in each well.

4 When the gel has polymerized (approximately 30 minutes), place gel in 1X TBE buffer and remove combs. Load each well with a mixture of 15ul PCR product (from step 2) with 3ul of 6X gel loading buffer. Load ~15ul of ladder in separate lane.

5 Run the gel at 85V for ~30 to 45 minutes

6 Expected fragment size:

Exon 1: 214 base pairs

Exon 2: 356 base pairs

Exon 3: 323 base pairs

Exon 4: 306 base pairs

Exon 5: 350 base pairs

7 PCR fragment purification:

The individual PCR fragments are excised, purified using the QIAquick PCR Purification Kit Protocol (Qiagen, Valencia, CA USA), and submitted for automated DNA sequencing.

8 DNA Sequence analysis:

DNA sequences derived from individual PCR fragments are compared to the normal expected sequences via the basic local alignment search tool (BLAST), National Center for Biotechnology Information, U.S. National Library of Medicine.

Basic Protocol 9: ATOX1 Gene Mutation Analysis

The ATOX1 gene encodes a protein that acts as a copper chaperone, transporting cytosolic copper to ATPases. The analysis is performed by PCR amplification of coding sequences of an individual's DNA, followed by purification and direct sequencing of the PCR fragments.

Materials

~300ng/ul genomic DNA (isolated from whole blood using Wizard Genomic DNA Purification Kit)

ATOX1 oligonucleotide primers at 10uM (5' – 3'):

Exon 1 Forward: AGGCGCTGCTGACACCGCCG

Exon 1 Reverse: TTCAAGATCAGCATCCGGTC

Exon 2 Forward: AGGCTTCTGATGAGTCTGATGC

Exon 2 Reverse: TCTGCATGCATCTGAACATG

Exon 3 Forward: TGAGTAGTAATTTAGAGCCTG

Exon 3 Reverse: AGGTGTTCGCTCTGATGAGAG

10 mM dNTP mix (Invitrogen)

5 U/ul Taq DNA Polymerase High Fidelity (Invitrogen)

10X High Fidelity PCR Buffer (Invitrogen)

50 mM MgSO₄

PCR water

NuSieve agarose (Lonza)

1X TBE Buffer

10 mg/ml Ethidium Bromide Solution (Invitrogen)

6X gel loading buffer

GeneRuler DNA Ladder Mix (Fermentas)

Protocol

1 Set up PCR amplification reactions in PCR tubes for final volume of 50ul:

1ul template genomic DNA

2 ul ATOX1 forward and reverse primers (10uM each)

5ul 10X High Fidelity PCR Buffer

1ul 10mM dNTP mixture

2ul 50mM MgSO₄

.2ul Platinum Taq High Fidelity DNA Polymerase

38.8ul PCR water

2 Perform PCR using the following protocol:

Initial step:	3min 94°C (denaturation)
35 cycles:	30s 94°C (denaturation)
	30s 57°C (annealing)
	1.5min 72°C (extension)

Final step: Store at 4°C 7min 72°C

- 3 Prepare a 2% agarose gel solution by slowly adding 1g of agarose to 50ml of 1X TBE. Mix thoroughly and heat in a microwave twice for 30 second intervals, swirling the mixture after each heating. Let solution cool slightly and add 2ul of ethidium bromide. Swirl to completely mix the solution. Immediately cast in appropriate size gel tray. Insert combs in order to load 20ul in each well.
- 4 When the gel has polymerized (approximately 30 minutes), place gel in 1X TBE buffer and remove combs. Load each well with a mixture of 15ul PCR product (from step 2) with 3ul of 6X gel loading buffer. Load ~15ul of ladder in separate lane.
- 5 Run the gel at 85V for ~30 to 45 minutes
- 6 Expected fragment size:
 - Exon 1: 120 base pairs
 - Exon 2: 272 base pairs
 - Exon 3: 326 base pairs
- 7 PCR fragment purification:

The individual PCR fragments are excised, purified using the QIAquick PCR Purification Kit Protocol (Qiagen, Valencia, CA USA), and submitted for automated DNA sequencing.
- 8 DNA Sequence analysis:

DNA sequences derived from individual PCR fragments are compared to the normal expected sequences via the basic local alignment search tool (BLAST), National Center for Biotechnology Information, U.S. National Library of Medicine.

9. Commentary

a. Background Information

Disorders of Human Copper Metabolism: An Overview: To date, four genes have been implicated in human disorders of copper metabolism: *ATP7A*, *ATP7B*, *CP*, and *CCS*. Three distinct and well-characterized disorders related to mutations in *ATP7A* have been described: Menkes disease (Kaler et al., 1992; Liu et al., 2005; White et al., 1992), occipital horn syndrome, and *ATP7A*-related distal motor neuropathy. The neurological, biochemical and molecular features of these three disorders are summarized in recent reviews (Møller and Tümer, 2010; Kaler, 2011).

Mutations in *ATP7B* are responsible for Wilson disease (Schilsky, 2009; Kaler, 2010), an autosomal recessive condition of hepatic copper overload with neurologic manifestations. Aceruloplasminemia is technically a disorder of iron metabolism, but features low serum copper and ceruloplasmin levels. This condition leads to progressive neurodegeneration of the retina and basal ganglia, and marked accumulation of iron in affected parenchymal tissues (Harris et al., 1998). Mutations in *CCS*, the chaperone to copper superoxide dismutase, may contribute to a clinical syndrome of congenital cataracts, deafness, neurodevelopmental delays, and liver disease, associated with low serum copper and ceruloplasmin (Huppke et al., 2010).

Principle of HPLC-EC: *HPLC* The HPLC-EC system works on the principle that analytes of interest are injected into the fluid path that carries the mobile phase through the system. The mobile phase is pumped at a constant rate over a stationary phase (the analytical column). The analytes are eluted from the column separately because of their characteristic retention times.

Electrochemical Detection: After the analytes of interest have been separated via the analytical column, quantitation of the amounts of the analytes is based on the strength of the current generated from application of oxidation/reduction potentials in the post-column conditioning cell containing a flow-through electrode and an analytical cell containing two flow-through electrodes. The first electrode in the series is set at an oxidizing potential and the third at a reducing potential, so that only reversibly oxidized species are detected. The output from the third cell is recorded by a Data Acquisition System such as a Waters Empower system.

The detailed techniques described in this chapter have evolved over the past nearly 50 years when Menkes disease was first recognized as a copper transport disorder. Spawned by identification of the genes responsible for Menkes disease and Wilson disease, the past two decades have witnessed a remarkable growth in our knowledge and understanding of eukaryotic copper metabolism. Appreciation of the basic pathways that guide cellular copper uptake, transport and export has reached a reasonable level; however, considerably less is known about the precise mechanisms that underlie the neurological consequences of disturbed copper homeostasis, and the ideal remedies. The diagnostic assays recounted here have been reliable over many years for the diagnosis of known copper transport diseases. The DNA sequence-based methods for analyzing other copper transport genes should facilitate identification of important new genotype-phenotype correlations.

b. Critical Parameters—For plasma catechol testing in newborns, since the neurochemicals involved are temperature-sensitive, proper sample collection, storage and shipment are critical to obtain valid results. Blood from patients should be collected into chilled sodium heparin tubes and immediately placed on wet ice. Within 2 hrs, samples should be spun down in a refrigerated centrifuge and the plasma frozen immediately on dry ice. If stored before shipping, it should be at -80 degrees C since catechols stored at -20 degrees C are not stable. Finally, the specimens must be shipped on sufficient dry ice to prevent thawing during transit.

The fibroblast ^{64}Cu pulse-chase experiments for diagnosis of Menkes disease rely upon healthy cells, and a source of radiolabelled copper with high specific activity.

The prenatal diagnosis of Menkes disease by CVS copper content requires use of stainless steel instruments in excellent condition and not contaminated by copper. Stainless steel instruments, while not 100% copper-free, contain mainly iron and chromium. Unless the instrument surface becomes degraded, leaching of copper from instrument to tissue should be unlikely.

c. Troubleshooting—For failures in PCR, consider fresh reagents including newly synthesized primers. DNA contamination in PCR is common, and segregation of workstations for PCR setup and analysis, respectively, is essential. For example, PCR products should never return to the PCR set-up region. Separate dedicated pipette sets for PCR set-up and electrophoretic analysis of PCR products should be maintained.

d. Anticipated Results—The diagnostic results obtained with these techniques are highly reliable for the diagnoses of Menkes and Wilson disease, and Aceruloplasminemia.

Alterations in CCS, CTR1, and ATOX1 should be interpreted in the context of patients' clinical and biochemical phenotypes. *In silico* analyses of putative mutations in all the genes can be performed using PolyPhen-2 (**P**oly**m**orphism **P**henotyping **v**2), a tool which predicts the possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations. (See <http://genetics.bwh.harvard.edu/pph2/>)

e. Time Considerations—The assays described in this module can all be completed within one week's time, or less.

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Figure 1. Basis for Diagnosis of Menkes Disease by Plasma Catechols

Compounds that are proximal metabolites in the catecholamine biosynthetic pathway accumulate to supranormal levels whereas distal metabolites are lower than normal. DOPA: dihydroxyphenylalanine; DOPAC: dihydroxyphenylacetic acid; DBH: dopamine beta hydroxylase; DHPG: dihydroxyphenylglycol

Figure 2. Chromatograph of alumina eluate from catechol standards

Abbreviations: DHPG=dihydroxyphenylglycol; NE=norepinephrine; DOPA=dihydroxyphenylalanine; EPI=epinephrine; DHBA=dihydroxybenzylamine; DA=dopamine; DOPAC=dihydroxyphenylacetic acid. A total of 250 pg each of NE, EPI, and DA and 1000 pg each of DHPG, DOPA, and DOPAC were subjected to batch alumina extraction before injection into the liquid-chromatographic-electrochemical system.

Figure 3. Chromatograph of alumina eluate of plasma from a newborn at-risk of Menkes disease
Abbreviations as for Figure 2. Normal DOPAC:DHPG, DOPA:DHPG, and DA:NE ratios exclude Menkes disease.

Figure 4. Chromatograph of alumina eluate of plasma from a newborn with Menkes disease
Abbreviations as for Figure 2. Elevated DOPAC:DHPG, DOPA:DHPG, and DA:NE ratios indicate decreased conversion of DA to NE via dopamine- β -hydroxylase and therefore support a diagnosis of Menkes disease.

Figure 5. Copper transport studies in cultured fibroblasts

Scatter plot of ^{64}Cu quantity and percent retention after 24 hr “chase” period shows clean separation between patients affected with Menkes disease (red diamonds, $n=44$) and unaffecteds (yellow squares, $n=17$).

A	P104-110510_59802-N_B02			
	Probe Name	Bin Size	P104-110510_59802-N_B02	
1	Xp11.4	274.0	0.960	
2	Xp21.1	301.0	1.003	
3	Xp22.1	327.5	1.000	
4	Xp22.31	220.0	1.000	
5	Xp22.32	247.5	1.000	
6	Xp22.33	416.5	1.000	
7	Xq23	381.0	0.930	
8	Xq23.1	133.0	0.960	
9	Xq25	356.0	1.004	
10	Xq28	190.5	1.021	
11	Xq28	443.5	1.000	
12	Xq28.1	166.5	1.035	
13	ex01a	149.0	1.000	
14	ex01b	348.0	1.000	
15	ex02	176.0	1.000	
16	ex02b	436.0	1.000	
17	ex03	203.0	1.009	
18	ex04	363.5	0.976	
19	ex05	256.0	0.998	
20	ex06	280.0	0.978	
21	ex07	228.0	1.000	
22	ex08	398.5	1.000	
23	ex09	311.5	1.025	
24	ex10	340.0	0.979	
25	ex11	371.5	1.000	
26	ex12	158.0	0.953	
27	ex13	184.0	0.945	
28	ex14	408.0	1.064	
29	ex15	212.0	1.010	
30	ex16	197.0	0.984	
31	ex17	238.5	1.000	
32	ex18	391.5	0.974	
33	ex19	265.5	1.000	
34	ex20	291.0	1.000	
35	ex21	140.0	1.001	
36	ex22	321.5	0.990	
37	ex23	426.0	1.000	

B	P104-110510_52257_E02			
	Probe Name	Bin Size	P104-110510_52257_E02	
1	Xp11.4	274.0	1.011	
2	Xp21.1	301.0	0.993	
3	Xp22.1	327.5	0.936	
4	Xp22.31	220.0	0.975	
5	Xp22.32	247.5	1.037	
6	Xp22.33	416.5	0.944	
7	Xq23	381.0	1.016	
8	Xq23.1	133.0	0.935	
9	Xq25	356.0	1.035	
10	Xq28	190.5	1.024	
11	Xq28	443.5	0.914	
12	Xq28.1	166.5	1.012	
13	ex01a	149.0	0.978	
14	ex01b	348.0	0.948	
15	ex02	176.0	1.017	
16	ex02b	436.0	0.978	
17	ex03	203.0	1.000	
18	ex04	363.5	1.049	
19	ex05	256.0	0.000	
20	ex06	280.0	0.000	
21	ex07	228.0	0.000	
22	ex08	398.5	0.000	
23	ex09	311.5	0.000	
24	ex10	340.0	0.000	
25	ex11	371.5	0.000	
26	ex12	158.0	0.000	
27	ex13	184.0	0.000	
28	ex14	408.0	0.000	
29	ex15	212.0	0.000	
30	ex16	197.0	0.000	
31	ex17	238.5	1.004	
32	ex18	391.5	1.015	
33	ex19	265.5	0.991	
34	ex20	291.0	0.970	
35	ex21	140.0	0.985	
36	ex22	321.5	1.026	
37	ex23	426.0	0.997	

Figure 6. Output of MLPA assay for alterations in ATP7A

The relative peak areas (column 4) of each probe are shown in a normal control (A) and a Menkes disease patient with deletion of exons 5–16 (shaded region in column) (B).

Initial step:	5min 95°C (denaturation)
35 cycles:	30s 95°C (denaturation)
	30s x°C (annealing)
	y s 72°C (extension)
Final step: Store at 4°C	7min 72°C

Table 1

Primers for PCR amplification, ATP7A gene:

Exon	Primers (L:Forward R:Reverse)	Sequence Lower letters:universal tags*, upper letters:gene specific	Expected fragment size (base pair)
1	MNKex1L	accactgcttactggcttaccATCACCCGGAAGTGGG	385
	MNKex1R	gaggggcaaacaacagatggcCGAGACTGCCCCAGACTAAC	
2	MNKex2L	accactgcttactggcttaccCAACTTTCCTTTACTTCATCTAAGG	501
	MNKex2R	gaggggcaaacaacagatggcTTGCCAAATGTGTCAAGTGTG	
3	MNKex3aL	accactgcttactggcttaccTCAAATGCCTGATAGGTACCAC	683
	MNKex3aR	gaggggcaaacaacagatggcCTTCAGCAGCACTTCACCAG	
	MNKex3bL	accactgcttactggcttaccATTGCTGAAGACCAAGGGTG	442
	MNKex3bR	gaggggcaaacaacagatggcCTCTAGTCTCATGGATTCT	
4	MNKex4aL	accactgcttactggcttaccTTTCTAGTCTATGCCAATAACATTTTC	559
	MNKex4aR	gaggggcaaacaacagatggcTCACAATGGCAGACCTATTCTC	
	MNKex4bL	accactgcttactggcttaccTCACAGCAAAGGAGTCCATC	404
	MNKex4bR	gaggggcaaacaacagatggcTGCCATCAATGTTTATCACAG	
	MNKex4cL	accactgcttactggcttaccATAGAGGCTGTATACCGGG	528
	MNKex4cR	GaggggcaaacaacagatggcCTGCTGGCTACCTCCATAGG	
5	MNKex5L	accactgcttactggcttaccGGAATAGATTGTCAGTGCCTGG	472
	MNKex5R	gaggggcaaacaacagatggcTGAAAATCTTTATGAGGCCTTACC	
6	MNKex6L	accactgcttactggcttaccTTCTCCAGATTCAAATCCTTAAATAC	468
	MNKex6R	GaggggcaaacaacagatggcCCTTCCATTTTCCAGTTTCC	
7	MNKex7L	accactgcttactggcttaccTGGTAACTCATGTTTAAATGGTGG	341
	MNKex7R	GaggggcaaacaacagatggcAACCAGAAATGATTCACGGG	
8	MNKex8L	accactgcttactggcttaccCCAGAGTGACTTGCCCTCAG	358
	MNKex8R	gaggggcaaacaacagatggcGATATACATTGTGACCATTTCATCC	
9	MNKex9L	accactgcttactggcttaccTGGATGAAATGGTCACAATG	496
	MNKex9R	gaggggcaaacaacagatggcGCATATGGCATAAAATGATCAGAC	
10	MNKex10L	accactgcttactggcttaccCGATTGTTTTGGCTTAATCTCC	548
	MNKex10R	gaggggcaaacaacagatggcTGAAAATACGTCCTTCAGTAAATGG	
11	MNKex11L	accactgcttactggcttaccAAAGCTGTCTTTTGGGTAAATG	383
	MNKex11R	gaggggcaaacaacagatggcTGAAGTATGGCAAAATGTGG	
12	MNKex12L	accactgcttactggcttaccTGGTTCTAGCTTCCATTGGC	462
	MNKex12R	gaggggcaaacaacagatggcAACTAAAGCCACAAAGTAAATCTGAG	
13	MNKex13L	accactgcttactggcttaccTTTACCTAGGCATTGTTCAG	444
	MNKex13R	gaggggcaaacaacagatggcAAACGAACTCCATATTAATGCAAC	
14	MNKex14L	accactgcttactggcttaccCACCACACCTGGCCATTAAC	440
	MNKex14R	gaggggcaaacaacagatggcTCACTCTCCACTCCAAACC	
15	MNKex15L	accactgcttactggcttaccTTCTGGAATCTCAGTATGTCCC	628
	MNKex15R	gaggggcaaacaacagatggcAACAAAGGCCAATCTGAAGC	

Exon	Primers (L:Forward R:Reverse)	Sequence Lower letters:universal tags*, upper letters:gene specific	Expected fragment size (base pair)
16	MNKex16L	accactgcttactggcttattcGTTCAGCATTGGCAGAGGAG	667
	MNKex16R	gaggggcaaacaacagatggcTGTCAACAGGAGTTATCTGTGTG	
17	MNKex17L	accactgcttactggcttattcATCAAAATCCACTGTCAAGTAGG	534
	MNKex17R	gaggggcaaacaacagatggcGGCATAGGGTATTGACTTGAGG	
18	MNKex18L	accactgcttactggcttattcACAATGTAGTGAAGTGTGCAAGG	403
	MNKex18R	gaggggcaaacaacagatggcTTCAAGATGCTTTTGAATAACCC	
19	MNKex19L	accactgcttactggcttattcTTAAGTTAAGCCATGCCCTG	460
	MNKex19R	gaggggcaaacaacagatggcAAGCATGGTAAATTTCTTGAAGG	
20	MNKex20L	accactgcttactggcttattcATGTGGACATCTTGTGGGTG	467
	MNKex20R	gaggggcaaacaacagatggcAATGGCTGATAGCATGGAAC	
21	MNKex21L	accactgcttactggcttattcGCTGAGTCTAAGTTTATAGCATTATCTG	450
	MNKex21R	gaggggcaaacaacagatggcAACATTACCTCTCACCATAACCAG	
22	MNKex22L	accactgcttactggcttattcCCACCAAGAGGATAAATGCTG	373
	MNKex22R	gaggggcaaacaacagatggcAATGGTTTGGGCTTATCATTG	
23	MNKex23L	accactgcttactggcttattcCCACACAATTCGTTTCAAGC	629
	MNKex23R	gaggggcaaacaacagatggcTGCCAACAGATAAACGCAAC	