Automated Spectrophotometric Analysis of Mitochondrial Respiratory Chain Complex Enzyme Activities in Cultured Skin Fibroblasts

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Background: Mitochondrial respiratory chain complex (RCC) disorders may occur as commonly as 1 in 8500 individuals. Because of the great variability of phenotypic presentations, measurement of individual RCC enzyme activities is a crucial diagnostic process. Current assay methods are time-consuming and labor-intensive and thus constitute a major impediment to clinical practice. A method with a faster turnaround time would therefore be beneficial.

Method: We developed an automated spectrophotometric method for measuring the respiratory chain enzyme activities of complex I, complex II/H11545 III, and complex IV with the Hitachi 912, an automated spectrophotometer. Mitochondrial citrate synthase was also determined for normalization of the RCC activities.

Results: A blinded method comparison with samples from an external testing center yielded a 91% concordance of interpretations. Mean intraassay imprecision (as CV; n = 20) in a single batch analysis of each RCC was 5.9%. Interassay imprecision, evaluated on 2 samples harvested and analyzed 3 times each, gave mean CVs of 10%–18%.

Conclusions: With this automated method, a panel of RCC enzyme activities can be determined in <2 h. In addition, an immunoblot assay using monoclonal antibodies against specific subunits of RCC enzyme complexes can be informative in cases of borderline enzyme activity. Our results suggest that in vitro diagnosis of RCC enzyme deficiencies in skin fibroblasts is an effective alternative to invasive muscle biopsy.

The mitochondrial respiratory chain complexes (RCCs)5 coenzyme Q, and cytochrome c catalyze energy transduction from respiratory substrates to a proton motive gradient for the synthesis of ATP. This pathway is made up of 5 multienzymatic complexes (complexes I–V). These complexes are encoded by both the nuclear genome and mitochondrial DNA (mtDNA); therefore, pedigrees with either autosomal or maternal inheritance can be observed. In addition, sporadic cases of RCC deficiencies are also encountered.

Catalytic defects in this pathway can be tissue-specific, necessitating the development of diagnostic enzyme assays applicable to a variety of cells and tissues. Limitations of these methods that may lead to inconclusive results include the lack of an appropriate control group, insufficient operator experience, and the absence of standardized methods for measuring RCC enzymes (1). Moreover, RCC assays are usually performed with time-consuming manual methods, and the techniques are available in only a small number of specialized laboratories.

To facilitate the clinical process, diagnostic classification of adult cases has been introduced (2). More recently, diagnostic criteria based on clinical, metabolic, imaging, and histopathologic features have been proposed for infants and children to complement the results of biochemical investigations of skeletal muscles (3). Bernier et

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5 Nonstandard abbreviations: RCC, respiratory chain complex; mtDNA, mitochondrial DNA; CS, citrate synthase; PVDF, polyvinylidene difluoride; PBS, phosphate-buffered saline; and PBST, PBS plus Tween 20.
al. (4) attempted to merge the diagnostic criteria in adults and children, improving the sensitivity of the adult criteria. Despite these efforts, the diagnosis of an RCC defect is still challenging, because normal respiratory enzyme activity in a particular tissue (e.g., fibroblasts) does not exclude an underlying mitochondrial defect, even when the initially tested tissue (e.g., muscle) clinically exhibits the disease. In addition, deficient respiratory enzyme activity does not imply that oxidative phosphorylation is primarily impaired.

Use of a random-access analyzer for automated analysis of mitochondrial enzymes of cytochrome c oxidase (complex IV) and citrate synthase (CS) in cultured skin fibroblasts was first described by Williams et al. (5). This automated method was faster to perform, less expensive, and required less than one half of the sample material needed for traditional manual methods. Determination of complex III (ubiquinol:ferrocytochrome c oxidoreductase) activity by an automated protocol with reliable reproducibility and precision was described recently (6).

In response to a recent study suggesting that mitochondrial RCC disorders may occur as commonly as 1 in 8500 individuals (7) as well as rapidly growing clinical demand, we developed and validated a panel of RCC automated assays that allow the simultaneous determination of all RCCs in cultured skin fibroblasts on a widely available spectrophotometric analyzer. To compare our automated method with the classic manual methods, we collaborated with The Hospital for Sick Children (Toronto, Canada) for cross-validation. We also investigated an immunologic approach to determine whether the analysis of protein concentrations coupled with an automated assay can provide a unique perspective into the basis of mitochondrial defects.

**Materials and Methods**

NADH, EDTA, dithionitrobenzoic acid, oxaloacetic acid, MgCl₂, potassium phosphate, KCN, potassium ferricyanide, sodium borohydride, antimycin A, rotenone, and succinic acid were purchased from Sigma. Bovine serum albumin was purchased from Serologicals Corporation. Protein analysis was performed with the Micro TP test (Wako). Decylubiquinone (BioMol) was prepared according to a previously described method (8) and stored protected from light at −70 °C before analysis. Cytochrome c (from bovine heart) was purchased from Sigma. Reduced cytochrome c was prepared fresh on each day of analysis as follows: A 1 mmol/L solution was prepared in 20 mmol/L potassium phosphate buffer (pH 7.2), and was reduced with sodium borohydride. After reduction, 2 drops of 2 mol/L HCl were added to precipitate excess sodium borohydride, and the supernatant was incubated at 37 °C for 20 min to stabilize the solution. For electrophoresis and membrane transfer, NuPAGE 4%–12% Bis-Tris Novex gels with MES buffer, transfer buffer, and an XCell SureLock apparatus were purchased from Invitrogen. Transfer membrane, composed of 0.45 μm polyvinylidene difluoride (PVDF), was purchased from Millipore. For Western blotting, a monoclonal antibody cocktail of anti-ND6, anti-complex II 30-kDa, anti-complex III core2, anti-complex IV subunit 2, and anti-porin, were purchased from Mitosciences. Secondary goat anti-mouse, alkaline phosphatase–conjugated antibodies and dried milk powder for blocking were purchased from Bio-Rad. Alkaline phosphatase developing solution was purchased from Invitrogen.

**CELL CULTURE AND HARVEST**

This study was approved by the Mayo Foundation Institutional Review Board committee. Skin fibroblasts were cultured and harvested by previously established methods (9) with some modifications. Normal control cell lines were purchased from Coriell and were grown until confluent in 1 T-75 flask with MEM containing 200 μmol/L uridine, nonessential amino acids, and 100 mL/L fetal bovine serum. After trypsinization, fibroblast cells were washed with phosphate-buffered saline (PBS; 137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L NaHPO₄, 1.4 mmol/L KH₂PO₄), and the suspension was centrifuged at 400g for 5 min. The wash and centrifugation steps were repeated twice more before the fibroblasts were resuspended in PBS and centrifuged at 7200g for 2 min. The resulting pellet was resuspended in 20 mmol/L phosphate buffer (pH 7.2) and sonicated on ice for two 5-s bursts in a Sonicator Ultrasonic Processor XL (Heat Systems Inc) at 10% power. The preparation was centrifuged a final time at 600g for 10 min, and the supernatants were kept on wet ice until analysis.

Clinical validation was performed with cultured skin fibroblasts from patients with an established defect of complex I (n = 7), complex I + III (n = 1), or complex IV (n = 2) and from a control (n = 1). The cell lines were sent to our laboratory for blind analyses from the Hospital for Sick Children in Toronto, and the results and interpretations were compared after all analyses had been completed.

**AUTOMATED ANALYSIS**

Protein concentrations and respiratory chain enzyme activities were determined on a Hitachi 912 (Roche Diagnostics). This instrument is a robotic spectrophotometer with 120 semipermanent cuvettes and a 70-specimen capacity. It automatically transfers samples and reagents to a cuvette, incubates them at 37 °C, and reads the absorbance at a specified wavelength. Each programmed assay can use up to 4 reagents, designated R1–R4. Reagents designated as R1 are dispensed into the cuvette 10 s after the sample protein. Reagents R2, R3, and R4 are dispensed 1.5, 5, and 10 min, respectively, after R1. Absorbance readings are taken every 20 s, and calculations can be programmed into the instrument to automatically transform reaction rate data into enzyme activities.

Fibroblast homogenates were diluted to 1 g/L total protein with 20 mmol/L potassium phosphate buffer
(pH 7.2) before RCC analysis. Automated assay conditions were based on previously published spectrophotometric methods (5, 8, 10–12).

Assay conditions were as follows: complex I (rotenone-sensitive NADH-CoQ reductase; OMIM 252010; EC 1.6.5.3) activity was determined with two different 4-min, 2-point rate assays: one that measured the total complex I activity and one that measured rotenone-insensitive complex I activity. These assays measured the oxidation of NADH at 340 nm, with a secondary wavelength of 415 nm (ε = 6.81 mmol L⁻¹ cm⁻¹). To determine the total activity, 19 µL of fibroblast homogenate was combined with 350 µL of a pH 7.2 substrate solution (R1) containing 2.66 g/L bovine serum albumin, 27 mmol/L potassium phosphate buffer, 0.213 mmol/L NADH, 5 mmol/L MgCl₂, 0.1065 mmol/L decylubiquinone, 0.002 g/L antimycin A, and 2 mmol/L KCN. The decrease in absorbance was monitored. Rotenone-insensitive complex I activity was measured by use of the same substrate solution to which 39.9 µmol/L rotenone was added. Fibroblast homogenate (19 µL) was combined with 350 µL of the substrate, and the decrease in absorbance was again monitored. The difference between the total and rotenone-insensitive activity rates was used to calculate the complex I rotenone-sensitive enzyme activity.

We measured the activity of complex II + III (succinate cytochrome c reductase) with a 5-min, 2-point rate assay after the reduction of cytochrome c at 546 nm with 570 nm as a reference wavelength (ε = 19.1 mmol L⁻¹ cm⁻¹). Fibroblast homogenate (50 µL) was added to 300 µL of a pH 7.8 substrate solution (R1) containing 25 mmol/L succinic acid, 37.5 mmol/L potassium phosphate, 0.5 mmol/L EDTA, 0.005 g/L rotenone, and 1.87 mmol/L KCN. After 1.5 min, 37 µL of 0.5 mmol/L cytochrome c (R2) was added. The monitored increase in absorbance was used to calculate the complex II + III enzyme activity.

A 15-min, 2-point rate assay that followed the oxidation of cytochrome c at 546 nm and a secondary wavelength of 570 nm was used to measure complex IV activity (cytochrome oxidase; OMIM 516030, 516040, and 516050; EC 1.9.3.1; ε = 19.1 mmol L⁻¹ cm⁻¹). Fibroblast homogenate (37 µL) was incubated for 5 min with 328 µL of 40 mmol/L potassium phosphate, pH 7.2 (R1), before the addition of 5 µL of 1 mmol/L reduced cytochrome c (R3). The decrease in absorbance was monitored continuously for 5 min to observe the linearity, and the initial rate was estimated by measuring the first 1.66 min of the complex IV activity reaction. Reagent 4 [5 µL; 10 mmol/L K₃[Fe(CN)]₆] was then added, and the absorbance was observed for an additional 5 min.

CS (OMIM 118950; EC 2.3.3.1) activity was measured with a 15-min, 2-point rate assay at 415 nm, with a secondary wavelength of 450 nm (ε = 13.6 mmol L⁻¹ cm⁻¹). Fibroblast homogenate (19 µL) was incubated for 5 min with 318 µL of 1 mol/L potassium phosphate buffer, pH 7.2 (R1), before addition of 30 µL of a solution containing 2.5 mmol/L dithionitrobenzoic acid and 2.5 mmol/L acetyl-CoA (R3). After incubation for 5 min, 7 µL of 10 mmol/L oxaloacetate (R4) was added. The increase in absorbance was used to calculate CS enzyme activity.

For all assays, the enzyme activity was calculated by the following equation:

\[ \frac{\Delta A}{min} \times \text{dilution factor} \times \frac{1}{L} \times \frac{1}{0.6 \text{ cm}} \times \frac{1000 \mu g}{mg} \times \frac{1}{\mu g/\mu L} = \text{enzyme activity/mg protein} \]

where \(\Delta A/\text{min}\) is the change in absorbance per minute; 0.6 cm is the spectrophotometer cell pathlength; and \(\epsilon\) is molar absorptivity (in mmol·L⁻¹·cm⁻¹).

Assay performance was monitored by corun analysis of 3 control cell lines and a blank specimen consisting of fibroblast homogenate that had been heat-inactivated in a boiling water bath for 5 min.

**Western Blot Analysis**

Approximately 7 µg of fibroblast proteins, dissolved in sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer, were resolved on NuPAGE 4%–12% Bis-Tris Novex gels with MES buffer in an XCell SureLock apparatus at 150 V. After electrophoresis, proteins were transferred to 0.45 µm PVDF membranes in Invitrogen transfer buffer for 2 h at 0.2 A, or for 16.5 h at 0.035 A, at 4 °C. The PVDF membranes were blocked with 50 g/L nonfat dried milk powder in PBS, 0.5 mL/L Tween 20 (PBST) for 30 min at room temperature. After blocking, PVDF membranes were incubated with both primary antibodies, 3 mg/L OXPHOS cocktail and 1 mg/L antiporin, diluted in PBST containing 10 g/L dried milk powder for 2 h at room temperature or overnight at 4 °C. Membranes were washed 3 times with PBST and incubated with 0.3 mg/L alkaline phosphatase–conjugated goat anti-mouse IgG (heavy and light chain) antibody in PBST containing 10 g/L milk for 1 h at room temperature. After PVDF membranes were rinsed twice with PBST and PBS, the blots were developed with alkaline phosphatase color development buffer (0.1 mol/L diethanolamine, 5 mmol/L MgCl₂, 0.3 g/L nitroblue tetrazolium chloride, and 0.15 g/L 5-bromo-4-chloro-3-indolyl-phosphate), and the color reaction was stopped by washing with H₂O. Alkaline phosphatase signals were quantified by scanning dried blots with an HP Scanjet and quantifying band pixels with ImageQuant 5.2 software. Ratios of individual proteins were calculated in relation to porin signals. Control fibroblasts porin signals were set to 100%, and patient cell lines were reported in comparison with this value.

**Results**

**Reference Ranges**

To determine reference ranges for the components of the RCC profile, 14 cell lines from Coriell cell repository were each analyzed multiple times for RCC activity. The resulting data are shown in Table 1. The mean rotenone-sensitive enzyme activity was calculated from the following equation:

\[ \frac{\Delta A}{min} \times \text{dilution factor} \times \frac{1}{L} \times \frac{1}{0.6 \text{ cm}} \times \frac{1000 \mu g}{mg} \times \frac{1}{\mu g/\mu L} = \text{enzyme activity/mg protein} \]
sensitivity was 30% of total complex I activity (range, 15%–50%).

**PRECISION**

Intraassay imprecision was determined for each RCC by 20 replicate analyses. The activity data (Table 2) yielded a mean CV of 5.9%. Interassay imprecision was evaluated for 2 samples, harvested and analyzed 3 times each (Table 3). Mean CV values were 13% for complex I, 18% for complex II + III, 13% for complex IV, and 10% for CS.

**METHOD COMPARISON**

The clinical specificity of the automated method was assessed by blinded comparison of results from 10 patients with established diagnoses and 1 healthy control from the Hospital for Sick Children in Toronto, where RCC activity measurements are performed as a clinical service (Table 4). The results from the 2 facilities were concordant for the diagnosis of complex II + III deficiency (patient 10) and for complex IV deficiency (patients 8 and 9). However, the complex IV enzyme rate reductions were less when measured at the Mayo Clinic. For complex I deficiency, the severe enzyme deficiencies measured at the Hospital for Sick Children were replicated at the Mayo Clinic for patients 1, 2, and 4. Severe complex I deficiency was also diagnosed in patient 5, but the Mayo Clinic enzyme rate of 64% did not concur with the 33% deficiency compared with reference values obtained at Toronto. However, in this case, the complex I:CS ratio was very low (52% compared with reference values), indicating possible deficiency. The subunits of the enzyme measurements are demonstrated in patients 2 and 3, who had the same mutation in the ND5 subunit of complex I. The difference in diagnosis (deficient complex I activity for patient 2 and normal complex I activity for patient 3) was not reflected by the heteroplasmy of the mutation; importantly, however, our enzyme measurements were still concordant with those from the Hospital for Sick Children. Although the number of confirmed complex I–deficient cases was limited in this study, the mean complex I:CS ratio was close to 50%, which we considered as a cutoff for deficiency. Overall, concordance of interpretive results was achieved in 91% of specimens in the comparison.

**WESTERN BLOTTING**

In conjunction with the analysis of fibroblast cell lines by the automated activity assay, a second-tier immunologic assay was adopted to further improve the diagnosis of RCC deficiencies. Monoclonal antibodies that are highly specific for mitochondrial respiratory chain proteins are available commercially and have been described previously (13). With simple electrophoresis and Western blotting techniques, it is possible to examine the relative expression of specific mitochondrial proteins within crude fibroblast extracts. A monoclonal antibody cocktail of anti-ND6, anti-complex II 30-kDa, anti-complex III core 2, anti-complex IV subunit 2, and anti-porin was used to detect the relative expression in patient fibroblast after activity assay experiments. As shown in Fig. 1, an obvious distinction between clearly deficient (patients 4, 6, 8, and 9) and control fibroblasts can be readily assessed. This result was reproducible as determined by 4 different experiments. In some circumstances, Western blotting of patient fibroblast samples can provide additional insight into respiratory chain–deficient status that may go undetected by enzymatic assays. For example, the Western blot for patient 6 was from a sample that was scored within the reference range by the activity assay. However, it is clear that compared with controls, this sample contains a distinctly diminished amount of complex I subunit ND6. The Hospital for Sick Children determined the complex I enzyme rate to be only mildly deficient, and this patient’s diagnosis was based on complex I + III:CS ratio. The cause of this discrepancy is under investigation. There was also a presumably compensatory increase of complex

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**Table 1. Mean activity and ratio in control fibroblasts.**

<table>
<thead>
<tr>
<th>Complex</th>
<th>Mean (SD) activity, nmol · min⁻¹ · (mg protein)⁻¹</th>
<th>Mean (SD) ratio of activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>36.8 (11.2)</td>
<td>0.93 (0.34)</td>
</tr>
<tr>
<td>II+III</td>
<td>14.9 (3.2)</td>
<td>0.34 (0.13)</td>
</tr>
<tr>
<td>IV</td>
<td>24.4 (3.0)</td>
<td>0.74 (0.11)</td>
</tr>
<tr>
<td>CS</td>
<td>40.1 (5.8)</td>
<td>1.2 (0.17)</td>
</tr>
</tbody>
</table>

**Table 2. Assay performance: Intraassay imprecision.**

<table>
<thead>
<tr>
<th>Complex</th>
<th>Mean (SD) activity, nmol · min⁻¹ · (mg protein)⁻¹</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>37.0 (3.6)</td>
<td>9.7</td>
</tr>
<tr>
<td>II+III</td>
<td>15.6 (1.2)</td>
<td>7.5</td>
</tr>
<tr>
<td>IV</td>
<td>9.2 (0.5)</td>
<td>5.3</td>
</tr>
<tr>
<td>CS</td>
<td>37.0 (0.5)</td>
<td>1.2</td>
</tr>
</tbody>
</table>

**Table 3. Assay performance: Interassay imprecision.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>RCC activity, nmol · min⁻¹ · (mg protein)⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>52.2 16.6 28.1 35.1</td>
</tr>
<tr>
<td>B</td>
<td>33.3 15.5 25.0 26.5</td>
</tr>
</tbody>
</table>

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II subunit, comparable to the enzyme activity of complex II as 140% of mean control. The results for the 2 complex IV–deficient patients were consistent with the enzyme assay (patients 8 and 9 in Fig. 1). Overall, Western blotting identified 4 RCC-deficient patients from 8 validation samples tested.

The well-known difficulty of diagnosing complex I deficiency by Western blotting arises because the mutation of certain subunits may or may not prevent the complex from assembling partially or wholly. ND6, the subunit probed in these Western blots, is thought to be essential for assembly of the complex. However, for the 2 patients with ND5 mutations (patients 2 and 3 in Fig. 1), their deficiencies did not appear to affect the assembly of the complex, and hence the presence of the ND6 subunit on the blot. This lack of influence on complex I assembly has been identified before in human cell line mutants with frameshift mutations in the ND5 gene (14).

The validation samples used to demonstrate specific RCC subunits results within the reference range via Western blotting may contain a deficiency in a protein not probed, alterations in complex assembly, or diminished

### Table 4. Comparison of results for validation samples.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Clinical and laboratory findings</th>
<th>HSC diagnosis</th>
<th>Results from Mayo Clinic</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lactic acidosis; failure to thrive; hypertonic; lack of assembly of CI by Western blotting; increased L/P ratio; CI+III, 33%</td>
<td>CI</td>
<td>CI, 34% I/CS, 22% ND</td>
</tr>
<tr>
<td>2</td>
<td>G13513A mtDNA mutation in ND5 subunit of CI; ~80% mutant heteroplasmy as determined by DNA digest; increased L/P ratio; CI+III, 33%; CI+III:CS, 0.5</td>
<td>CI</td>
<td>CI, 36% I/CS, 48% I, 120%</td>
</tr>
<tr>
<td>3</td>
<td>G13513A mtDNA mutation in ND5 subunit of CI; ~87% mutant heteroplasmy as determined by DNA digest; increased L/P ratio; CI+III, 131%; CS, 132%; CI+III:CS, 0.97</td>
<td>CI</td>
<td>CI, 78% I/CS, 60% I, 124%</td>
</tr>
<tr>
<td>4</td>
<td>Lactic acidosis, hernias, ambiguous genitalia, syndactyly; increased L/P ratio; CI+III, 21%</td>
<td>CI</td>
<td>CI, 22% I/CS, 18% I, 0%</td>
</tr>
<tr>
<td>5</td>
<td>Hepatomegaly with renal tubulopathy, chronic liver disease, rickets, proximal renal tubular acidosis, developmental delay, FTT; increased L/P ratio; CI+III, 33%</td>
<td>CI</td>
<td>CI, 64% I/CS, 52% ND</td>
</tr>
<tr>
<td>6</td>
<td>A3260G mtDNA mutation in tRNA leucine; CI+III, 73%; CI+III:CS, 0.68</td>
<td>CI</td>
<td>CI, 89% I/CS, 70% I, 9%</td>
</tr>
<tr>
<td>7</td>
<td>Transient neonatal lactic acidosis, left esotropia; brother with CI deficiency; CI+III, 68%</td>
<td>CI</td>
<td>CI, 57% I/CS, 54% I, 195%</td>
</tr>
<tr>
<td>8</td>
<td>Deteriorating neurologic and cardiovascular course; increased L/P ratio; CIV, 6%</td>
<td>CIV</td>
<td>CIV, 21% IV/CS, 15% IV, 0%</td>
</tr>
<tr>
<td>9</td>
<td>Leigh disease; increased L/P ratio; CIV, 9%</td>
<td>CIV</td>
<td>CIV, 27% IV/CS, 13% IV, 0%</td>
</tr>
<tr>
<td>10</td>
<td>Progressive cerebral degeneration with atrophy, mental and motor regression, left hemiparesis and seizures; CI+III, 12%</td>
<td>CII+III</td>
<td>CII+III, 19% II+III/CS, 29% ND</td>
</tr>
</tbody>
</table>

* Enzyme activities shown as percentages of mean normal activities. L/P, lactate/pyruvate; CI, complex I; CI+III, complex I+III; FTT, failure to thrive; CIV, complex IV; CII+III, complex II+III.

* HSC, Hospital for Sick Children; ND, not detected.

* Enzyme activities shown as percentage of mean normal activity.

* Ratios of individual proteins were calculated in relation to porin signals from control fibroblasts set to 100%.

* Control complex I+III:CS ratio is ~0.75; ratios are shown only if CS rates were available.

Fig. 1. Western blot of total fibroblast proteins from validation cell lines and a fibroblast control.

Additional information about validation samples is listed in Table 4. An equal amount of protein (7 μg/lane) was loaded for the control cell line and for patients 8, 9, 4, 2, 6, 3, and 7. Expression of RCC subunits complex III core 2 (III), complex II 30-kDa (II), complex IV subunit 2 (IV), and complex I subunit ND6 (I) was detected by blotting with specific monoclonal antibodies (see Material and Methods). Porin was also detected with a monoclonal antibody and was included as a loading control.
enzyme function resulting from the disruption of superstructure or posttranslational modification.

Discussion
As mitochondrial disorders have become more widely recognized, the number of patients confirmed to be affected with a respiratory chain defect has increased almost exponentially. Greater clinical appreciation of these disorders has evolved despite limitations in current diagnostic approaches and the need for consensus diagnostic criteria (15). The Mira S (Roche) has been used for automated assessment of complex IV (5) and complex III activity (6), and we have developed a panel of mitochondrial RCC enzyme assays that use the Hitachi 912, a robotic spectrophotometer that handles the preparation and analysis of up to 70 samples at a time. This analyzer is typically used for high-throughput assays such as the determination of serum cholesterol and triglycerides.

Our automated assay for RCCs allows separate and group measurement of enzyme activities by use of specific electron acceptors and donors. This method does not require the isolation of a mitochondrial fraction and can be carried out with tissue homogenates. One T-75cm² flask of cultured fibroblasts provides a sufficient number of fibroblasts for measuring the whole panel of RCC enzymes.

According to Williams et al. (5), automated analysis of 25 samples for CS, cytochrome c oxidase, and lactate dehydrogenase took 3 h compared with 50 h for manually performed assays. Excluding the time taken to culture cells, the run time of our assay for the RCC panel with a similar number of samples is < 2 h. The small size of the reaction cuvettes saves sample and reagents, and the 70-sample capacity of the Hitachi 912 sample wheel could dramatically improve the turnaround time for the RCC enzyme assay. The observation of 91% concordant results in this study strongly supports the reliability of our automated method.

The rationale supporting fibroblast testing is based on the frequent performance of skin biopsies in patients faced with a broad range of differential diagnoses, particularly when there is a need to exclude both a fatty acid oxidation disorder and a respiratory chain defect (16). Permission to perform a muscle biopsy may not be granted by parents concerned about the invasive nature of the procedure, and the specimen is rarely sufficient for a repeat analysis. Such issues and objections are not commonly applicable to cultured fibroblasts.

The rotenone-sensitive NADH quinone reductase assay in skin fibroblasts has assumed greater importance in the investigation for RCC defects. One pitfall to the diagnostic use of this reaction is the presence of high rotenone-resistant activity in skin fibroblasts, as evidenced by recent studies demonstrating that isolated complex I deficiency is a major contributor to the group of respiratory chain defects (17, 18). In a study of isolated complex I deficiency in children (19), the enzyme activities in skin fibroblasts corresponded well to the results obtained with muscle tissues and aided in the identification of novel nuclear gene mutations. A recent study of 20 patients with complex I deficiency also suggested that the complex I deficiency in that group of patients is most probably caused by a defect in one of the nuclear encoded structural genes of complex I or in one of the genes involved in proper assembly of the enzyme (20). The studies by our coauthors (21) indicated that whole-cell [2-14C]pyruvate oxidation could be used as a complementary assay for the investigation of complex I defects in skin fibroblasts. It is also very important to note that pediatric mitochondrial disorders may be accompanied by normal muscle morphology, normal mitochondrial enzymes in skeletal muscle, and normal mtDNA mutation screening results (18). Thus, there is not a single tissue type that can completely reflect the status of all mitochondrial deficiencies. Considering this and the ability to obtain a skin biopsy during an outpatient visit, fibroblast testing early in the diagnostic process for patients with possible RCC defects has an advantage over muscle-based assays because for those who have the defect in skin fibroblasts, at least one half of all patients, an invasive procedure to obtain a sufficient volume of muscle tissue can be avoided (22). It is conceivable that with current diagnostic criteria or methods, test results for some patients with mitochondrial respiratory chain defects could be interpreted as normal; therefore, careful diagnosis must include clinical information and image studies in addition to other laboratory results. It is of note that even in our validation samples, 4 cell lines contained enzyme activities > 50% of our mean control. The Western blot study, however, was informative in one of these cases, indicating that the immunologic study should be a part of the investigation for mitochondrial RCC disorders in fibroblasts. Currently, we interpret the results as abnormal when the activity or ratio is < 50% of the control mean, but this cutoff may be adjusted as our understanding of mitochondrial RCC disorders increases. For now, the results should be interpreted cautiously in the context of clinical and other laboratory findings, particularly for patients with borderline deficiency.

We cannot yet offer an explanation for the discrepant enzyme activities in 2 patients with the same mutation, G13513A (patients 2 and 3 in Table 4). The heteroplasmy of the 2 cell lines with G13513A mtDNA mutations should not vary over time or with passage number. The experiment to determine whether other, subtle factors are affecting complex I activity or assembly is in progress.

In summary, we have developed an automated assay for measuring a full panel of RCC enzymes. This method has good precision, provides rapid results, and allows analysis of the whole panel of RCC enzymes from a small sample. Furthermore, our immunologic assay has shown additional benefits when used in conjunction with automated enzyme assays. By decreasing turnaround time for...
results and providing a noninvasive diagnostic option, our method could contribute to improved patient care.

References