itated proteins to avoid the rapid oxidation of SH groups (14), which takes place after neutral pH is restored for the DTT reduction and mBrB conjugation.

Measured basal GSSPs in human and rat RBCs are very low, and only a few previous reports found relatively close values (23). Conversely, most authors reported higher concentrations; one reason for these discrepancies could be that samples were frozen before the ESI-MS determination. It is possible that ferric Hb, hemicromes, and hemochromes, which can be generated by freezing (24), produce oxidants, increasing the Hb-SSG content. Our measures indicate that human and rat Hbs, under typical conditions, are minimally glutathionylated (9.9 nmol/g; i.e., 0.03% of β chains). A negligible percentage of GSH was found bound to membrane proteins. Hb-SSG content can increase after oxidative stress produced by diamide or t-BOOH treatments. In any case, human RBC GSSP content accounts for <10% of total cellular glutathione under oxidative stress. Conversely, rat Hb was shown to produce large amounts of Hb-SSG; after treatment with diamide, all GSH was bound to Hb. This is attributable to the peculiarity of rat Hb (25), which is characterized by a highly reactive cysteine in position β125, in addition to cysteine 93, which is common to most mammalian Hbs. In diabetes, where oxidative stress is increased, we have measured enhanced Hb-SSG (648%); in addition, we have also evaluated the correlation of obtained values with other indicators of oxidative stress, i.e., protein carbonyls, isoprostanes, and malondialdehyde. Preliminary results (not shown) indicate a positive correlation with protein carbonyls and isoprostanes but not with lipid peroxidation products.

In conclusion, our studies suggest, as discussed previously (9), that Hb-SSG could be used as a clinical marker of oxidative status and, consequently, of pathologies whose development is strongly associated with oxidative stress. The availability of a simple, sensitive, and reproducible method to detect Hb-SSG could be useful both for diagnostic and therapeutic purposes.

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References


Adaptation of a Mitochondrial Complex III Assay for Automation: Examination of Reproducibility and Precision, Jim Minchenko,1,4 Andrew J. Williams,5 and John Christodoulou1,2 1(1) Department of Paediatrics and Child Health, University of Sydney, Sydney, 2006 Australia; 2Western Sydney Genetics Program, Children’s Hospital, Sydney, 2145 Australia; 3Department of Clinical Immunology, Royal Prince Alfred Hospital, Sydney, 2050 Australia; 4address correspondence to this author at: Western Sydney Genetics Program, Royal Alexandra Hospital for Children, Locked Bag 4001, Westmead, NSW 2145 Australia; fax 61-9845-1864, e-mail Johnc@chw.edu.au

Complex III (ubiquinol:ferricytochrome c reductase; EC 1.10.2.2) catalyzes the reduction of cytochrome c by ubiquinol (1, 2). The assay measures the reduction of cytochrome c as catalyzed by complex III at 550 nm (3) in the presence of reduced decylubiquinone (DBH) (2).
Despite numerous in vivo methods to screen for disorders of ATP production, biochemical assays for the respiratory chain (RC) remain the best way to identify and characterize this group of disorders (4). Although investigators have documented various methodologies to measure complex III in a variety of tissue types (3, 5–7), these are based on manual methods that are unsuitable for screening large numbers of samples. We recently automated mitochondrial and RC-specific enzyme (8) and protein (9) assays on a random access analyzer (Roche Mira S). The benefits of automation include enhanced speed and simplicity; savings in costs, including labor and reagents, and in the amount of sample needed; and improvements in precision. We adapted and automated the complex III assay (2, 6) on the Roche Mira S random access analyzer and compared methods of isolation, performed stability studies, and examined the precision of this new automated method.

Skin fibroblasts from healthy human controls and patients were grown from skin explants. Fibroblasts were cultured in DMEM (high glucose) supplemented with 100 mL/L fetal bovine serum and 225 μmol/L uridine (10).

Two procedures were used to isolate mitochondria from cultured fibroblasts, with all sample preparation being carried out at 4 °C. The first method used a glasson-Teflon homogenizer, based on the long method of Pütänen et al. (11), except that four confluent T75 flasks were used, and the final mitochondrial pellet was resuspended in 400 μL of the sucrose buffer. The comparative method was based on the use of 1 g/L digitonin for 35 s before centrifugation and resuspension of the pellet in MOPS buffer (12).

Samples prepared by either method were subjected to three freeze-thaw cycles and sonication (Branson sonifier 250; six pulses, using 30% duty cycle, output control 3), immediately before being assayed (10). For both extraction procedures, isolated mitochondrial extracts were snap-frozen and stored at −80 °C until ready for biochemical analysis.

Decylubiquinone (Sigma) was reduced to DBH₂ according to the method of Trounce et al. (2), with minor modifications. To 250 μL of 10 mmol/L decylubiquinone we added ~5 mg of potassium borohydride and 10 μL of 0.1 mol/L HCl. The DBH₂ was stabilized with 10 μL of 1 mol/L HCl.

The reagents used for the assay were as described by Rahman et al. (6), except that decylbenzylquinol was replaced with DBH₂ and the final reaction volume was 500 μL. All automated assays (including citrate synthase and protein) were performed at 37 °C on a Mira S automatic analyzer (Roche). For the automated complex III assay, the main reagent vessel contained potassium phosphate buffer, potassium cyanide, rotenone, bovine serum albumin, and maltoside (all obtained from Sigma). The start reagent 1 vessel contained DBH₂ and the start reagent 2 vessel contained ferricytochrome c.

Complex III (ubiquinol:ferricytochrome c oxidoreductase) activity was calculated based on the initial quasilinear rate. The reduction of ferricytochrome c was measured by the increase in absorbance at 550 nm after addition of the sample at cycle 13. The main reagent buffer was loaded into the cuvette in cycle 1 (426–431 μL). At cycle 13, sample was added (5–10 μL), followed by start reagent 1 (DBH₂; 6 μL) in cycle 14 and start reagent 2 (ferricytochrome c; 7.5 μL) in cycle 15. Absorbance readings were then taken until cycle 40, each cycle being 25 s. Absorbance readings were expressed as μmol · min⁻¹ · mg of protein⁻¹ and also relative to citrate synthase. Both citrate synthase and protein were also measured on the Mira S as reported by Williams et al. (8). Samples were measured in duplicate.

Extracted mitochondrial pellets from healthy controls and from patients with a defect in complex III (all assayed for complex III, citrate synthase, and protein) were kept under different conditions (duration and temperature) to determine the stability of the complex III enzyme and the repeatability of the assay. Digitonin- and mortar-and-Teflon pestle-extracted samples were kept at 4 °C for 1 week before being assayed, and aliquots were also kept at room temperature for 24 h before being assayed. In addition, samples underwent one freeze-thaw cycle for the intrarun comparison. The volume used in the complex III stability studies was 10 μL.

Agreement was assessed graphically by Bland–Altman plots (13). CVs and SDs for intra- and interrun precision comparisons were estimated from duplicate assays performed on samples.

For a given sample volume, the intra- and interrun variability was very similar for the comparisons of both isolation methods, with the intrarun difference in CV being <1% for 5 and 10 μL of test sample (Table 1). These results suggest that either method was suitable for isolating mitochondria-containing extracts from primary fibroblasts in culture.

When we compared 5- and 10-μL digitonin-extracted pellets, there was a threefold difference in the intrarun CV (Table 1). Bland–Altman plots (values not shown) of the

<table>
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<tr>
<th>Table 1. Comparison between sample volume, extraction methods used, and storage conditions.</th>
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<td>Method of mitochondrial pellet extraction*</td>
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<tr>
<td>Digiton</td>
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<tr>
<td>Intrarun CV, %</td>
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<td>5-μL sample</td>
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<td>10-μL sample</td>
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<td>Interrun CV, %</td>
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<td>Change in activity + after storage for</td>
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<td>24 h at room temperature</td>
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<td>24 h at 4 °C</td>
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<td>7 days at 4 °C</td>
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<td>7 days at −80 °C</td>
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*See text for details.

n, number of samples assayed.

+Percentage change in complex III activity under the specified conditions: (−), decrease in activity; (+), increase in activity.
difference between the duplicates vs the mean of complex III activity showed that for the 5-µL sample, the majority of measurements were scattered in the 10–20% range. Doubling the volume to 10 µL for the same digitonin-extracted samples gave improved reproducibility: the Bland–Altman plot showed that most samples had complex III activity measurements clustered between 5% and 10%. Mortar-and-pestle-isolated 5-µL (CV, 9.1%) and 10-µL (CV, 3.9%) mitochondrial extracts showed a comparable trend in reproducibility and precision (Table 1).

The effects of storage temperature and duration on complex III activity in isolated mitochondrial extracts are shown in Table 1. Loss of enzyme activity varied, depending on the extraction method, with digitonin-extracted samples showing greater losses of enzyme activity than mortar-and-pestle-isolated samples. As expected, samples stored at −80 °C for 7 days showed the least enzymatic deterioration.

Isolated complex III deficiency is less common than isolated defects in complexes I and IV of the RC (14), but it occurs more frequently as part of a multicomplex defect (4). Although automatic enzyme assays are commonplace in many diagnostic laboratories, laboratories are unlikely to invest time or resources in automating biochemical assays of RC enzymes because it is perceived that these disorders are relatively rare. However, recent studies suggest that mitochondrial RC disorders may be as common as 1 per 8500 individuals (15).

Automation of the complex III assay may seem unnecessary at first. We have been using cybrid studies to evaluate inheritance patterns in RC disorders (10), which requires isolation and evaluation of many clones (>20). Because complex III has subunits encoded by both nuclear and mtDNA gene(s) (16), incorporation of automated assay methods into cybrid studies saves both time and resources.

A comparison between the longer, traditional method (mortar and pestle) as described by Pitkänen et al. (11) and the quicker, crude (digitonin) whole-cell extract method used by Williams et al. (8) is shown in Table 1. Although the differences in within- and between-run imprecision are minimal, as evidenced by the differences in the CV being <1%, the crude (digitonin) method has the advantages of being faster and requiring less starting material.

A comparison of the intrarun CVs at the different sample volumes showed that 10-µL samples had a lower CV (one-third lower) than 5-µL samples; therefore, in subsequent studies we used only a 10-µL sample volume.

When compared with published methods for complex III assays (7), our method requires a much smaller volume (10 µL vs 500 µL) and proportion of test sample (2.5% vs 50%); in addition, reagent costs for our assay are one-half the costs for the published assays. With the crude (digitonin) extraction method, we were able to use less starting material (2 × 10-cm Petri dishes), thus saving time in both the culture of cells and in the preparation of the mitochondrial pellet.

The stability of the enzyme assay was demonstrated by the minimal loss of activity, especially when samples were stored at −80 °C for 7 days, which would allow batching of large numbers of samples in a single run.

In summary, we have developed a method that may permit faster, less expensive screening of test samples and be well suited to research programs where large numbers of assays must be performed rapidly without compromising precision.

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References