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Isolated deficiencies of OXPHOS complexes I and IV are identified accurately and quickly by simple enzyme activity immunocapture assays

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ABSTRACT

OXPHOS deficits are associated with most reported cases of inherited, degenerative and acquired mitochondrial disease. Traditional methods of measuring OXPHOS activities in patients provide valuable clinical information but require fifty to hundreds of milligrams of biopsy tissue samples in order to isolate mitochondria for analysis. We have worked to develop assays that require less sample and here report novel immunocapture assays (lateral flow dipstick immunoassays) to determine the activities of complexes I and IV, which are far and away the most commonly affected complexes in the class of OXPHOS diseases. These assays are extremely simple to perform, rapid (1–1.5 h) and reproducible with low intra-assay and inter-assay coefficients of variability (CVs) s (<10%). Importantly, there is no need to purify mitochondria as crude extracts of whole cells or tissues are suitable samples. Therefore, the assays allow use of samples obtained non-invasively such as cheek swabs and whole blood, which are not amenable to traditional mitochondrial purification and OXPHOS enzyme analysis. As a first step to assess clinical utility of these novel assays, they were used to screen a panel of cultured fibroblasts derived from patients with isolated deficiencies in complex I or IV caused by identified genetic defects. All patients (5/5) with isolated complex IV deficiencies were identified in this population. Similarly, almost all (22/24) patients with isolated complex I deficiencies were identified. We believe that this assay approach should find widespread utility in initial screening of patients suspected of having mitochondrial disease.

1. Introduction

Defects of the oxidative phosphorylation (OXPHOS) system are the cause of many different diseases such as Leigh's, MERRF, MELAS, NARP, and LHON, each individually rare, but with a combined incidence of about 1:5000 [1–3]. There are 5 large complexes involved in OXPHOS, namely NADH ubiquinone oxidoreductase or complex I, succinate ubiquinone oxidoreductase (complex II), ubiquinone cytochrome *c* oxidoreductase (complex III), cytochrome *c* oxidase (complex IV) and the ATP synthase (complex V). Isolated deficiencies in complex I (CI) are the most common followed by isolated deficiencies of complex IV (CIV) [4–7]. There are also genetic defects in which multiple OXPHOS complexes including CI and CIV are deficient resulting either from long deletions in the mtDNA, from point mutations in the several tRNAs in this genome required for protein synthesis within the organelle and in nuclear genes involved e.g. in mitochondrial maintenance and the mtDNA translation machinery.

Both CI and CIV are large multiprotein enzyme complexes composed of both mtDNA-encoded and nuclear DNA encoded subunits. Therefore, deficits in amount or enzyme functioning can result not only from mutations or damage to nuclear or mtDNA-encoded structural proteins, but also from alterations in nuclear DNA-encoded factors required for proper assembly of these enzyme complexes. Indeed, it has been reported that most cases of early-onset, inherited isolated CI and CIV deficiencies are caused by defects in complex-specific assembly factors and not in complex-specific structural proteins [8].

Detection and diagnosis of OXPHOS deficiencies has until now depended on an extensive evaluation of clinical presentation of patients along with blood and urine chemistry analysis, particularly for lactic acid levels and alanine and unexpected metabolic intermediates. Also done are activity measurements of the 5 OXPHOS complexes, usually from large biopsy of muscle or from fibroblasts taken from the patient and cultured to produce workable amounts for mitochondrial purification. Open biopsy is invasive, particularly in infants presenting with potential OXPHOS disease and requires general anesthesia [9]. More acceptable would be methods for measuring OXPHOS activities that do not require mitochondrial isolation and could be performed on samples obtained by non-invasive means.

We have recently developed a set of assays for determining the amount and activity of OXPHOS complexes that use monoclonal antibody immunocapture to isolate each complex for analysis. Several attributes provide these assays with the potential to be used in primary screening of patients with suspected OXPHOS deficiency. The

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assays are rapid, accurate and can be done on a few micrograms of cell extract. Therefore they can be done on samples obtained by much less invasive means than an open biopsy e.g. cheek swabs, urine sediment or blood. If a biopsy is necessary, for example because the defect is localized to muscle, needle biopsy provides sufficient material for analysis.

As a first step to evaluate the utility of these novel methods we have compared results obtained by the immunocapture assay approach with the previous gold standard assays obtained using intact mitochondria. To do this we have taken advantage of an important resource, a set of fibroblast cell lines from patients with defined OXPHOS deficiencies collected over several years by the team of excellent clinical investigators at the Nijmegen Centre for Mitochondrial Disorders, Radboud University Nijmegen Medical Centre, Netherlands. For each of these cell lines a clinical profile of the patient is available as well as OXPHOS activities measured spectrophotometrically on mitochondria isolated from each. We show that the dipstick assays work well to identify CIV and CI patients from controls in almost all (27 of 29) patients tested. There are 2 exceptions, which are discussed in detail later.

2. Materials and methods

2.1. Patients

The clinical, biochemical and genetic data from analysis of the patients' cultured fibroblasts is presented in Table 1. The study group was composed of 24 patients with isolated complex I deficiencies (values below the lowest control), 5 with isolated complex IV deficiencies (values below the lowest control) and 10 age-matched controls. Complex I and IV activities were measured as previously described [10,11].

Table 1
Patient data information

Patient #	Gene	Mutation	CI activity (mU/UCOX)	CIV activity (mU/UCS)
Complex I				
1	NDUFS2	P299Q	29	597
2	NDUFS2	S413P	44	2189
3	NDUFS2	R228Q	54	1163
4	NDUFS2	R228Q	nd	860
5	NDUFS2	nd	29	958
6	NDUFS2	F84L/E104G	71	650
7	NDUFS4	W97X	nd	nd
8	NDUFS4	W97X	65	681
9	NDUFS4	R106X	60	nd
10	NDUFS4	R106X	36	860
11	NDUFS4	VPEEH167/VEKSIstop	62	783
12	NDUFS4	K158fs	75	nd
13	NDUFS7	V122M	65	710
14	NDUFS8	P79L/R102H	80	870
15	NDUFS8	R94C	16	704
16	NDUFV1	R59X/T423M	70	1350
17	NDUFV1	A341V	50	1100
18	NDUFV1	A211V	nd	nd
19	NDUFV1	R59X/T423M	80	800
20	ND2	L71P	42	920
21	ND3	10191T>C	59	927
22	ND6	M63V	67	924
23	ND5	13513G>A	93	1174
24	ND5	13513G>A	78	1154
Complex IV				
25	SURF1	121	117	
26	SURF1	G124R	nd	<62
27	SURF1	INS470A	243	144
28	SURF1	326insATdelTCTGCCAGCC	133	104
29	COX10	M1T	169	220
Controls				
Mean			140 ($n=10$)	805 ($n=6$)
Range			114–190	620–1077

nd = not determined.

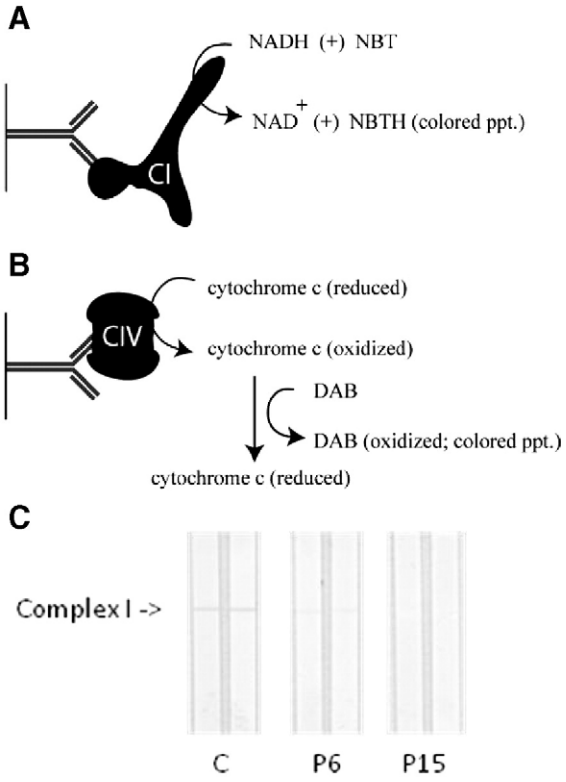

Fig. 1. Complex I and IV enzyme activity dipstick assays. (A) Complex I (CI) Activity dipstick reaction. Immunocaptured CI oxidizes NADH and reduces NBT, which forms a colored precipitate at the complex I immunocapture zone. The signal intensity is proportional to the concentration of enzymatically active complex I in the sample. (B) Complex IV (CIV) Activity dipstick reaction. Immunocaptured CIV oxidizes cytochrome *c*, which subsequently oxidizes DAB to form a colored precipitate at the complex IV immunocapture zone. The signal intensity is proportional to the concentration of enzymatically active CIV in the sample. (C) Representative images of CI activity dipsticks run (in duplicate) using samples taken from control fibroblasts (C) and patients with either partial (P6) or complete (P15) reduction in CI activity.

2.2. Whole cell protein extract preparations

Frozen fibroblasts cell pellets were thawed on ice and washed three times with 500 µl of PBS (pH 7.4). The cell pellet was then solubilized in 300 µl of Extraction buffer (1.5% n-Dodecyl-β-D-maltopyranoside (LM) (Anatrace, Maumee, OH), 25 mM Hepes (Sigma, St. Louis, MO) 100 mM NaCl (Sigma) with protease inhibitors (0.5 µg/ml leupeptin, 0.5 µg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride) for 20 min on ice. Following solubilization, the extract was clarified by centrifugation at 16,000 rpm for 20 min at 4 °C, the supernatant saved and stored frozen at −80 °C. Protein concentrations of the solubilized samples were determined by BCA analysis (Pierce, Rockford Ill.).

2.3. Monoclonal antibodies

Monoclonal antibodies (mAbs) that recognize native complex I were made by immunizing mice with sucrose gradient purified bovine heart complex I while anti-complex IV mAbs were made by immunizing mice with purified bovine heart mitochondria. The procedures for hybridoma preparation were as previously described [12], and the resulting hybridomas were screened for secretion of mAbs that specifically immunoprecipitate either complex I (mAb 18G12BC2) or complex IV (mAb 31E91B8) from both human and bovine heart mitochondria. The resulting immunoprecipitates were

characterized by mass spectrometric analysis as previously described to confirm specificity for complex I or complex IV [13,14].

The anti-frataxin lateral flow dipstick assays used here have been previously described [15] and were obtained from MitoSciences, Inc.

2.4. Enzyme activity quantitation dipstick assays

The complex I and IV enzyme activity dipsticks were prepared as shown in Fig. 1. First, a nitrocellulose membrane (Millipore, 12"×1.25") was affixed to an adhesive backing card (GL-187, 0.010" white matte vinyl) followed by placement of a cellulose wicking pad (Millipore #SA3J441H7) slightly overlapping the membrane. An Imagene Isoflow reagent dispenser (Imagene, Hanover, NH) was then used to dispense the capture mAbs in narrow zones across the width of the card. Complex I activity dipsticks contain a zone of anti-complex I mAb 18G12BC2 striped at 1 mg/ml while the complex IV activity dipsticks contain a zone of anti-complex IV mAb 31E91B8 stripped at 2 mg/ml (~18 μl/card). The striped cards were dried for 1 h at 37 °C and then stored in a desiccator chamber. Each card was then cut into 4 mm wide dipsticks using the Kinematic Matrix 2360 guillotine cutter (Kinematic, Twain Harte, CA).

Dipstick assays were performed by inserting individual dipsticks into 50 μl samples (25 μl of fibroblast extract mixed with 25 μl of 2× Sigma Block blocking buffer. The entire sample was then allowed to wick up laterally through the membrane, passing through the zone where the target enzymes are immunocaptured and concentrated (approximately 20 min). The dipsticks were then cleared by allowing 50 μl of wash buffer (50 mM Tris–Cl, 150 mM NaCl, pH 7.4) to wick up through the dipstick (approximately 15 min). The dipsticks were then transferred into the appropriate (CI or CIV) enzyme substrate buffer (described below) and enzyme activities calculated by measuring the optical density of precipitating, colorimetric enzyme reaction products. A Hamamatsu ICA-1000 immunochromatographic dipstick reader was used for densitometry.

Complex I activity was revealed and measured by immersing each complex I activity dipstick in 650 μl of complex I reaction buffer (20 mM Tris–Cl, pH 7.4 containing 0.1 mg/ml NADH (Sigma) and 0.3 mg/ml Nitrotetrazolium Blue (NBT, BioRad) for 30 min, stopping the reaction by rinsing with water and then measuring the amount of intensely colored, reduced NBT, which precipitates at the site of immunocapture of functional complex I. This functional enzyme assay for complex I is similar to in-gel activity assays for Complex I activity used for blue native gels [16,17].

Complex IV activity was measured by immersing each complex IV activity dipstick in complex IV reaction buffer (50 mM sodium Phosphate, pH 7.4 containing 0.1 mg/ml 3, 3′-diaminobenzidine (DAB) and 120 mM reduced cytochrome *c* (Sigma) for 60 min, stopping the reaction by rinsing with water and then measuring the amount of intensely colored, oxidized DAB that precipitates at the site of immunocaptured functional complex IV. This enzyme assay is similar to enzyme histochemical assays used to visualize complex IV activity in tissue sections [18].

2.5. Data analysis

All measurements were performed in duplicate and the results averaged. The mitochondrial protein frataxin was used to normalize loading as it has been shown that use of another mitochondrial protein as a loading control reduces variability of measured OXPHOS enzyme levels in both controls and patient samples [19].

Standard curves were made for each analyte (complex I activity, complex IV activity and frataxin protein levels) using a normal human dermal fibroblast control sample (outside of the control set subsequently analyzed) as a reference sample. The concentration of each analyte in every individual sample (patients and controls) was then determined by interpolating each raw densitometric dipstick value into the appropriate standard curve. The mean value for the study set of control samples was then calculated, set at 100% for each analyte and all individual samples were expressed as % of mean for the appropriate analyte.

3. Results and discussion

3.1. Enzyme activity quantitation dipstick assays measure levels of functional CI and CIV proteins accurately

The activity dipstick assays described here are lateral flow immunocapture assays that measure electron transfer activity of complex I and complex IV proteins. These tests are extremely simple, rapid (less than 1.5 h), accurate and reproducible. As shown schematically in Fig. 1, the capture mAb is immobilized in a zone on a nitrocellulose membrane backed on a plastic support. Samples are applied to the bottom of the dipstick and wick up laterally through the immunocapture zone. Target antigens (CI or CIV) in the sample are thus bound and concentrated in the immunocapture zone. Enzymatic activity of the captured enzyme is measured by incubating the

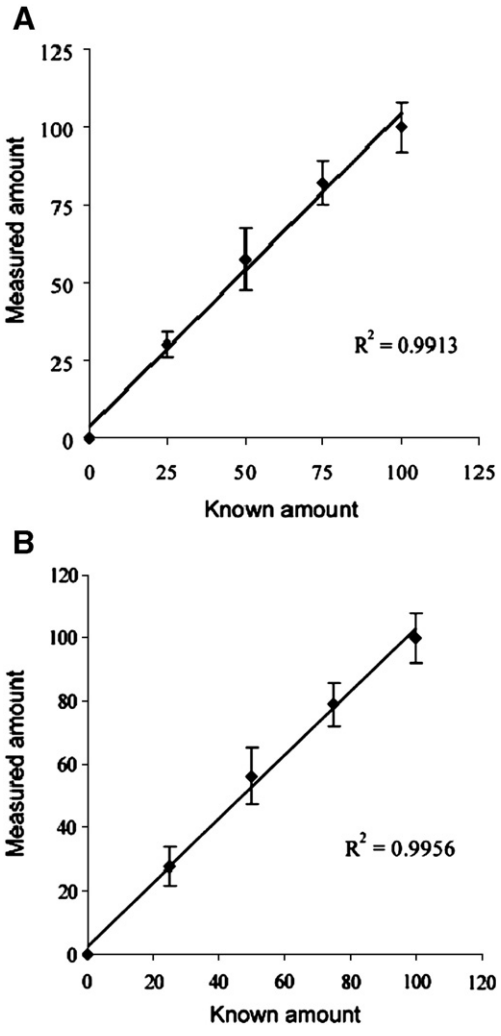

Fig. 2. Both complex I and IV enzyme activity dipsticks accurately measure levels of enzyme activities in defined blends of wild-type and ρ0 fibroblasts. Blends of wild-type dermal fibroblast (100% normal levels of both enzymes) and ρ0 dermal fibroblast (0% normal levels of both enzymes) whole cell extracts were made at precise increments and dipsticks were then loaded with equivalent amounts of total whole cell protein for every assay. Both CI (A) and CIV (B) enzyme activity values are nearly directly (1:1) proportional to the known amounts of assembled functional CI and CIV enzymes in the mixtures.

processed dipstick in a well containing CI or CIV enzyme reaction buffer as needed. Enzyme activity dipstick assays thus combine the specificity of immunocapture mAbs with the functionality of enzyme activity assays. The assays incorporate well-established enzyme activity assays commonly used for both in-gel [16,17], and histochemical [18] assays, using substrates that are both colorimetric and precipitating (see Materials and methods). The signal generated is proportional to the amount of active enzyme captured, which is turn is proportional to the amount of enzyme in the sample as long as saturating conditions are avoided.

3.2. Enzyme activity dipstick assays are accurate and reproducible

To assess accuracy, reproducibility and potential interference by other cellular proteins, each assay was tested using a sample set containing known levels of CI and CIV diluted in a background of heterogeneous proteins found in total cell extracts. A defined sample set was made by preparing a graded series of mixtures of wild-type human skin fibroblasts which contain normal levels of both CI and CIV (100% normal levels), and ρ0 human skin fibroblasts which completely lack these complexes (0% of normal). The ρ0 cells were prepared by growing normal skin fibroblasts in low levels of ethidium bromide for 10–12 population doublings, which results in complete depletion mtDNA [20]. Because both CI and CIV require a core of mtDNA-encoded subunits, ρ0 cells lack any fully assembled CI or CIV. This was confirmed by western blot and immunocytochemistry (not shown, but see reference [20]) and by dipstick measurements (Fig. 2).

A strong, direct (1:1) correlation was observed between the known and measured amounts of active enzyme (CIV: $R^2 = 0.9956$, CI: $R^2 = 0.9913$) (Figs. 2A and B) indicating that the assays measure their target antigens accurately and without interference from other proteins. In addition, both activity dipstick assays had intra-assay CVs < 10% throughout the working range ($n = 2$).

3.3. CIV enzyme activity dipsticks Identify all CIV patients tested

The sample set consisted of 5 patients with previously identified isolated deficiencies in CIV based on classical cytochrome *c* oxidase measurements that monitor reduction of cytochrome *c*. As controls, 6 fibroblast cell lines from unaffected individuals were included in the sample set for CIV analysis. As shown in Fig. 3A, the CIV activity dipstick assays readily distinguished the CIV patients from the controls. CIV patient cell lines had dipstick CIV activity values ranging between 7 and 30% of control mean, while in comparison control cell line values ranged from 40–164% of the control mean. Consistent with the positive identification of all CIV patients, the individual CIV activities measured by dipstick and traditional assays are highly correlated (Fig. 3B).

3.4. CI activity dipsticks identify 22 of 24 CI patients

The CI sample set contained 24 patients with previously identified isolated deficiencies in CI. The CI dipstick activity determination for each of these 24 CI patients is shown in Fig. 4, segregated by the gene in which the identified mutation is located, i.e., nuclear genes NDUFS2, NDUFS7, NDUFS8, NDUFS4, NDUFV1, and the mitochondrial encoded subunits. In all, 22 of the 24 CI patients had residual CI activities below that of the lowest control ($n = 10$, control range = 51–147% of the control mean). Again, the individual CI activities measured by dipstick and traditional assays correlate well (Fig. 4B) except for the cases noted below.

Notably, two CI patients had CI dipstick enzyme activities within the control range and each had a mutation in the mitochondrial encoded ND5 gene (Fig. 4). Thus, the NADH-NBT reductase dipstick assay failed to identify 2 patients with 13513 G>A mutations in ND5 although both cell lines had significantly reduced NADH-ubiquinone reductase activities as measured by the classical CI activity assay. ND5

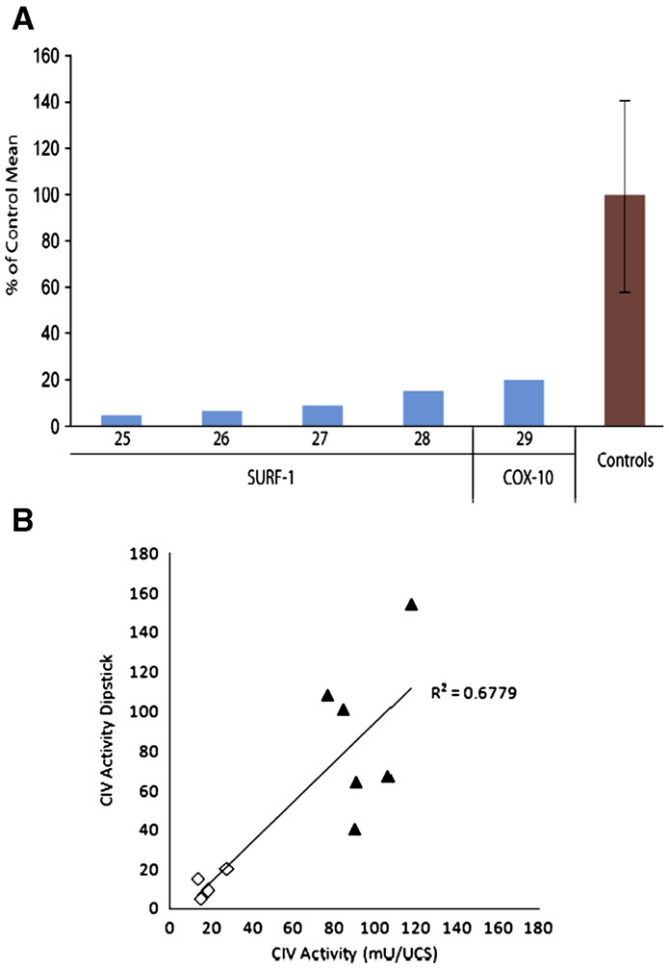

Fig. 3. CIV Enzyme activity dipsticks identify all patients with isolated CIV deficiencies. A) 5 of 5 patients with previously identified deficiencies in CIV exhibit CIV dipstick activity levels below the lowest control value. B) CIV activities measured by dipstick and traditional methods show a strong positive correlation ($R^2 = 0.6779$). Patients are organized by the gene name (see Table 1 for molecular lesion data). Two dipsticks per sample were analyzed, averaged and the relative levels of CIV activity were interpolated from a standard curve made using an independent control sample. Values from patients were expressed as percent of the control mean ($n = 6$). The range for controls was 40–164% of the control mean, which is shown +/− one standard deviation on the control bar. All dipstick measurements were normalized to frataxin protein quantity.

is an intrinsic membrane protein located in the core of the complex but thought not to be involved in ubiquinone binding and is without an intrinsic prosthetic group (non-heme iron center). However, the mutation clearly alters electron transfer to or through the ubiquinone site but evidently does not inhibit electron transfer through the flavin into the first few non-heme iron centers for passage to the electron acceptor NBT.

3.5. CI and CIV enzyme activity quantitation dipstick assays can be used to measure levels of CI and CIV in a wide range of clinically-relevant tissues

Because the enzyme activity dipstick assays are sensitive and do not require purification of mitochondria they can be used to measure levels of CI and CIV in a variety of clinically important tissue and cell types (Table 2). Most notable among these are samples collected by non-invasive means, including buccal swabs (cheek epithelial cells) and whole blood. A single cheek swab provides sufficient material to perform more than 100 tests while only microliter samples of whole blood are needed, amounts obtained easily by fingerprick sampling. Moreover, the ability to use whole tissue extracts as samples

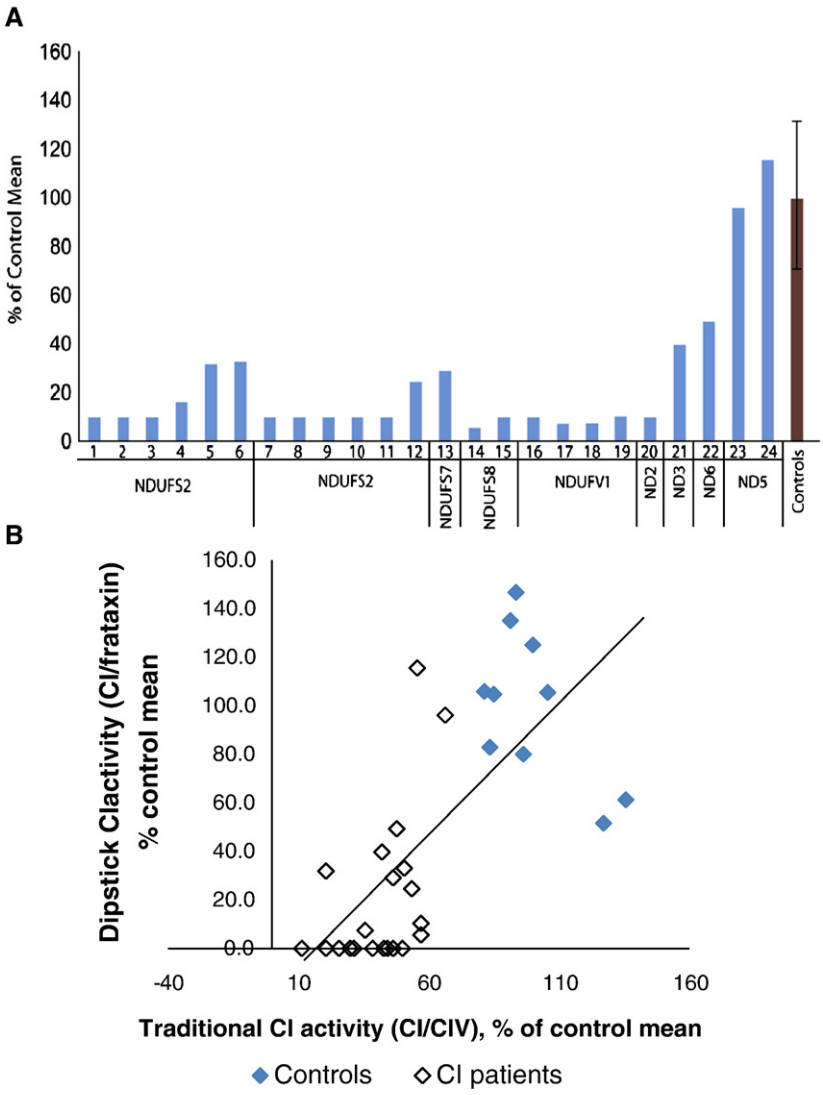

Fig. 4. CI enzyme activity dipsticks identify almost all (22 of 24) patients with isolated CI deficiencies. A) 22 of 24 patients with previously identified deficiencies in CI exhibit CI dipstick activity levels below the lowest control value. (B) CI activities measured by dipstick and the traditional, rotenone-sensitive assay for CI activity show a positive correlation ($R^2 = 0.494$). Patients are grouped by gene name and patient # (see Table 1 for more information). Two dipsticks per sample were analyzed, averaged and the relative levels of CI activity interpolated from a standard curve made using an independent control sample. Values from patients were expressed as percent of the control mean ($n = 10$). The range for controls was 51–147% of mean, which is shown +/− one standard deviation on the control bar. All dipstick measurements were normalized to frataxin protein quantity.

eliminates a significant source of potential false negative testing, namely the selective purification of healthy mitochondria during mitochondrial isolation procedures needed to prepare tissue samples for other types of analysis [21].

3.6. Prospects for using immunocapture based assays in diagnosis of OXPHOS diseases

Traditional OXPHOS enzyme assays provide important clinically-relevant information regarding mitochondrial function, but are complex and require sophisticated laboratory equipment and skilled personnel for proper performance. They are also difficult to standardize, making direct comparison of inter-lab test results problematic as demonstrated recently in a large comparative study in which multiple highly competent clinical labs assayed a standardized sample set, generating widely divergent results [22]. In contrast, the CI and CIV enzyme activity dipstick assays described here are simple, robust tools, well-suited for use as standardized diagnostic assays.

As shown above, the assays provide results comparable to traditional enzyme assays. The CIV dipstick activity assay was able to identify all 5 of the CIV patients identified previously by the more laborious traditional spectrophotometric CIV assay now used routinely as part of the evaluation of OXPHOS diseases. Similarly, although the CI dipstick activity assay measures only a partial activity, i.e., of electrons through the flavin and non-heme iron prothetic groups into NBT as an electron acceptor instead of passage through the entire complex and into ubiquinone, this simple assay identified 22 of 24 patients previously identified as CI deficient by the traditional ubiquinone reductase CI activity assay. Therefore, together the CI and CIV assays accurately detected 27 out of the 29 cases tested of a

Table 2
Dipstick enzyme activity assay working ranges for various human tissue types

Sample type	CI activity	CIV activity
Fibroblasts	2–30 μg	2–50 μg
Cheek cells (Buccal)	2–30 μg	2–50 μg
Whole blood	3–30 μl	3–30 μl
PBMC	2–30 μg	2–50 μg
Muscle tissue	0.5–10 μg	1–25 μg

Values shown are the amount of total cell protein or volume of whole blood loaded per dipstick test.

previously identified OXPHOS deficiency, or approximately 93% accuracy in comparison to the standard methods.

We have not considered the detection of mutations in complexes II, III or V in the present study, as they have been shown to account for only a small fraction of the OXPHOS defects identified to date, perhaps 5%. Taking these into account, together our dipstick assays for complex I and IV can be predicted to detect approximately 90% of all cases of OXPHOS enzyme deficits currently detectable by individual OXPHOS enzyme assays (93% accuracy of the 95% CI/CIV patients among the total). Given that the dipstick assays are so easy to perform, are reliable and require minimal amounts of sample, allowing measurement of CI and CIV activities in whole tissue extracts of non-invasive tissue samples such as buccal (cheek) swabs and whole blood, there is reason to believe that these two assays could be employed in an initial point of care screen that would be very valuable in diagnosis and management of patients. Although there is only limited data on the extent to which OXPHOS diseases are expressed in these easily accessible tissues, several recent studies indicate that OXPHOS abnormalities are reliably detected in them, with buccal samples being the more reliable sample when mtDNA mutation loads in each tissue are compared to those in skeletal muscle [23–25]. Further work is merited to assess the clinical utility of enzyme activity dipstick assays to detect OXPHOS deficits in patients using these tissues.

Recent clinical evaluation of a similar set of dipstick assays that measure the quantity (not activity) of assembled CI and CIV further demonstrates the promise of this approach [26]. In a study of the adverse metabolic effects of anti-HIV/AIDS drug therapy, we demonstrated that levels of assembled CI and CIV in peripheral blood mononucleated cells (PBMCs) parallel changes of CI and CIV levels in fat (a clinically affected tissue). Dipstick measurement of CI and/or CIV levels in easily sampled PBMCs could therefore serve as surrogate markers for OXPHOS deficits in peripheral fat which is itself only accessible by open biopsy [26].

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