PROTOCOL

MitoSciences
1850 Millrace Drive, Suite 3A
Eugene, Oregon 97403
www.mitosciences.com

Complex I Enzyme Activity Dipstick Assay Kit

MS130

Rev.2

DESCRIPTION

Complex I Enzyme Activity Dipstick Assay Kit

Sufficient materials are provided for 30 (MS130-30) or 90 (MS130-90) measurements.

Kit Contents:

Item	MS130-30	MS130-90
Dipsticks	30	90
Buffer A (Extraction buffer)	15 mL	45 mL
Buffer B (Blocking buffer)	2 mL	6 mL
Buffer C (Wash buffer)	1 mL	1 mL
Tube 1 (NBT – 50X stock)	200 µL	600 µL
Tube 2 (NADH – 100X stock)	100 µL	100 µL
Tube 3 (Buffer for activity assay)	10 mL	30 mL
96-well microplate	1	3

Storage:

Store dipsticks at room temperature in their provided container and out of direct sunlight. High humidity conditions should be avoided. Store Buffer A, B, and C at 4°C or at -20°C for long term storage. Store Tubes 1 and 2 at -80°C; they can also be aliquoted upon receipt to prevent freeze/thaw cycles. Tube 3 can be stored at room temperature.

INTRODUCTION

The Dipstick Assay Kit for Complex I (CI) Activity (MS130) is used to quantify the activity of Complex I (EC 1.6.5.3) from human, bovine, mouse and rat sample material. The isolation of mitochondria is not necessary for the performance of this assay. In this assay the specificity of MitoSciences' anti-Complex I monoclonal antibodies (mAbs) is combined with the well-characterized Complex I in-gel activity assay that is not rotenone sensitive. First, Complex I is immunocaptured (i.e. immuno-precipitated in active form) on the dipstick. Second, the dipstick is immersed in Complex I activity buffer solution containing NADH as a substrate and nitrotetrazolium blue (NBT) as the electron acceptor (Figure 1A). Immunocaptured Complex I oxidizes NADH and the resulting H^+ reduces NBT to form a blue-purple precipitate at the Complex I antibody line on the dipstick. The signal intensity of this precipitate corresponds to the level of Complex I enzyme activity in the sample (Figure 1B). Combined with MitoSciences' dipstick assay kit for measuring Complex I quantity (MS131 for human samples and MS133 for rodent samples), it is possible to determine the relative specific activity of immunocaptured Complex I. The signal intensity is best measured by a dipstick reader (MS1000, MitoSciences' Dipstick Reader) or may be analyzed by a standard imaging system.

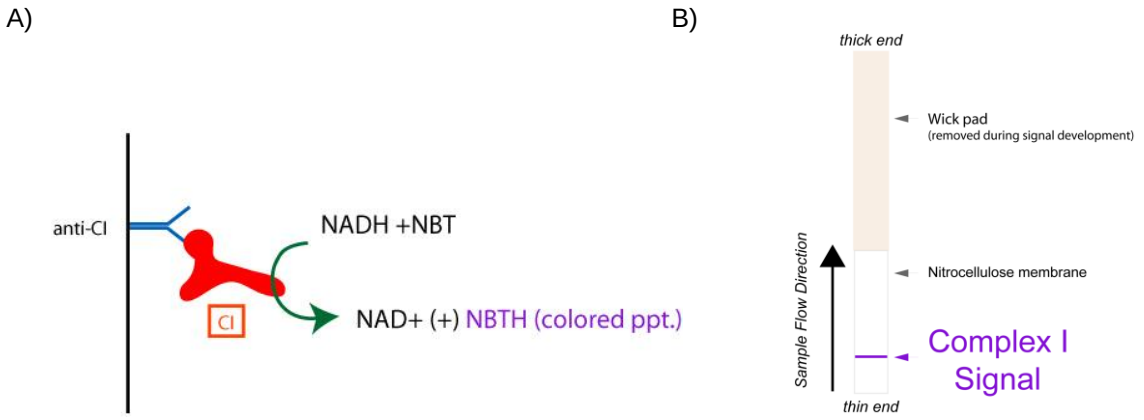


***Figure 1: Schematics of the Complex I activity dipstick reaction and a fully developed dipstick**. **(A)** Mechanism of the assay reaction; the dipstick with the immunocaptured Complex I is immersed in a solution containing NADH and NBT. Complex I oxidizes NADH which in turn reduces NBT to form a bluish-purple precipitate at the antibody line. **(B)** A developed Complex I Activity dipstick with the wicking pad removed. Note: The anti-Complex I mAb is located ~7 mm from bottom of dipstick. This reaction is not rotenone sensitive.*

ADDITIONAL MATERIALS REQUIRED

- Dipstick reader (MitoSciences' MS1000 Dipstick Reader) or other standard imaging system
- Method for determining protein concentration
- Pipetting devices
- Protease inhibitors

Telephone: 1-800-910-6486 or 541-284-1800 · Fax: 541-284-1801
www.mitosciences.com
Customer Service: sales@mitosciences.com · Tech Support: support@mitosciences.com

DIPSTICK ASSAY PROTOCOL

A. Sample Preparation

The preparation of the sample is a critical step in this procedure. The effective solubilization of the mitochondrial membranes in the sample is necessary. Please choose the sample preparation method that best fits your sample type. Follow each of the protocol steps carefully.

Note: *Samples must be kept on ice.*

1. Tissue samples:
    a. Begin with approximately 25 mg of sample.
    b. Add 5-10 volumes/ weight of iced Buffer A to the sample (e.g. if the total sample weight is 50 mg, add 250-500 µL of Buffer A).
    c. Homogenize the sample.
    d. Keep the sample on ice for 20 minutes and mix intermittently.
    e. Spin the cell extract in a microcentrifuge at 13,000 to 16,000 rpm for 20 minutes at 4°C.
    f. Remove the supernatant and determine the protein concentration of the protein extract from the sample. Protein concentration should be ≥ 1 mg/mL.
    g. Proceed directly to Part B of the Protocol or freeze samples at -80°C.
2. Cell culture samples:
    a. Add 5-10 volumes/ cell pellet volume of iced Buffer A to a cell pellet (e.g. if the cell pellet is 50 µL in volume, add 250-500 µL of Buffer A).
    b. Keep the sample on ice for 20 minutes and mix intermittently.
    c. Spin the cell extract in a microcentrifuge at 13,000 to 16,000 rpm for 20 minutes at 4°C.
    d. Remove the supernatant and determine the protein concentration of the protein extract from the sample. Protein concentration should be ≥ 1 mg/mL.
    e. Proceed directly to Part B of the protocol or freeze samples at -80°C.

Telephone: 1-800-910-6486 or 541-284-1800 · Fax: 541-284-1801
www.mitosciences.com
Customer Service: sales@mitosciences.com · Tech Support: support@mitosciences.com

B. Dipstick Procedure

This assay is most accurate with a user established standard curve for interpolation of signal intensity. Following the protein concentration ranges as defined in Table 1, generate a standard curve using a positive control sample (see example experiment on page 7 for reference).

Sample Type	Working Range
Fibroblast extract	1 - 30 µg
Skeletal muscle extract	0.5 - 15 µg
Bovine heart mitochondria	0.01 - 5 µg
Mouse tissue extract	0.05 - 5 µg
Rat tissue extract	0.05 - 5 µg

Table 1: *Suggested working range for different sample types.*

1. Load the amount of protein that corresponds toward the high end of the user generated standard curve (~3/4 of the high end).

2. Dilute samples to 25 µL with Buffer A.

3. Add 25 µL of Sample in Buffer A and 25 µL of Buffer B to a microplate well and mix.

   Note: If the protein concentration is too low, 100 µL reaction volumes are possible. Be sure to keep the ratio of sample in Buffer A to Buffer B constant.

4. Gently add a dipstick to the microplate well (place the thin/nitrocellulose end of dipstick down). The dipstick must reach the bottom of the well.

5. Allow the sample to wick up into the dipstick. This step takes 15-45 minutes depending on sample viscosity.

   Note: The entire sample volume has to be absorbed by the dipstick before proceeding to the next step, but do not allow the dipstick to dry at any time during this procedure.

6. Add 30 µL of Buffer C to each well with a dipstick.

7. Allow the dipstick to wick up the buffer for 10 minutes.

   Note: Do not allow the dipstick to dry out at any time.

8. Prepare the Activity Buffer by combining the contents of Tubes 1, 2, and 3 as per Table 2. Use 300 µL of Activity Buffer solution per dipstick.

Activity Buffer volume	Tube 1 (µL)	Tube 2 (µL)	Tube 3 (mL)
5 mL	100	50	4.85
10 mL	200	100	9.7

Table 2. *Preparation of Activity Buffer*

Telephone: 1-800-910-6486 or 541-284-1800 · Fax: 541-284-1801
www.mitosciences.com
Customer Service: sales@mitosciences.com · Tech Support: support@mitosciences.com

9. Add 300 µL of Activity Buffer to an empty microplate well for each dipstick used.

10. Remove the wicking pad from the dipstick. Make sure to remove the pad at the junction with the membrane.

11. Place the dipstick in a microplate well with Activity Buffer. The Complex I capture mAb is located ~7mm from the bottom of dipstick.

12. Develop for 30-45 minutes.

    Note: Since this is an end-point reaction, expose all samples to Activity Buffer for the same amount of time

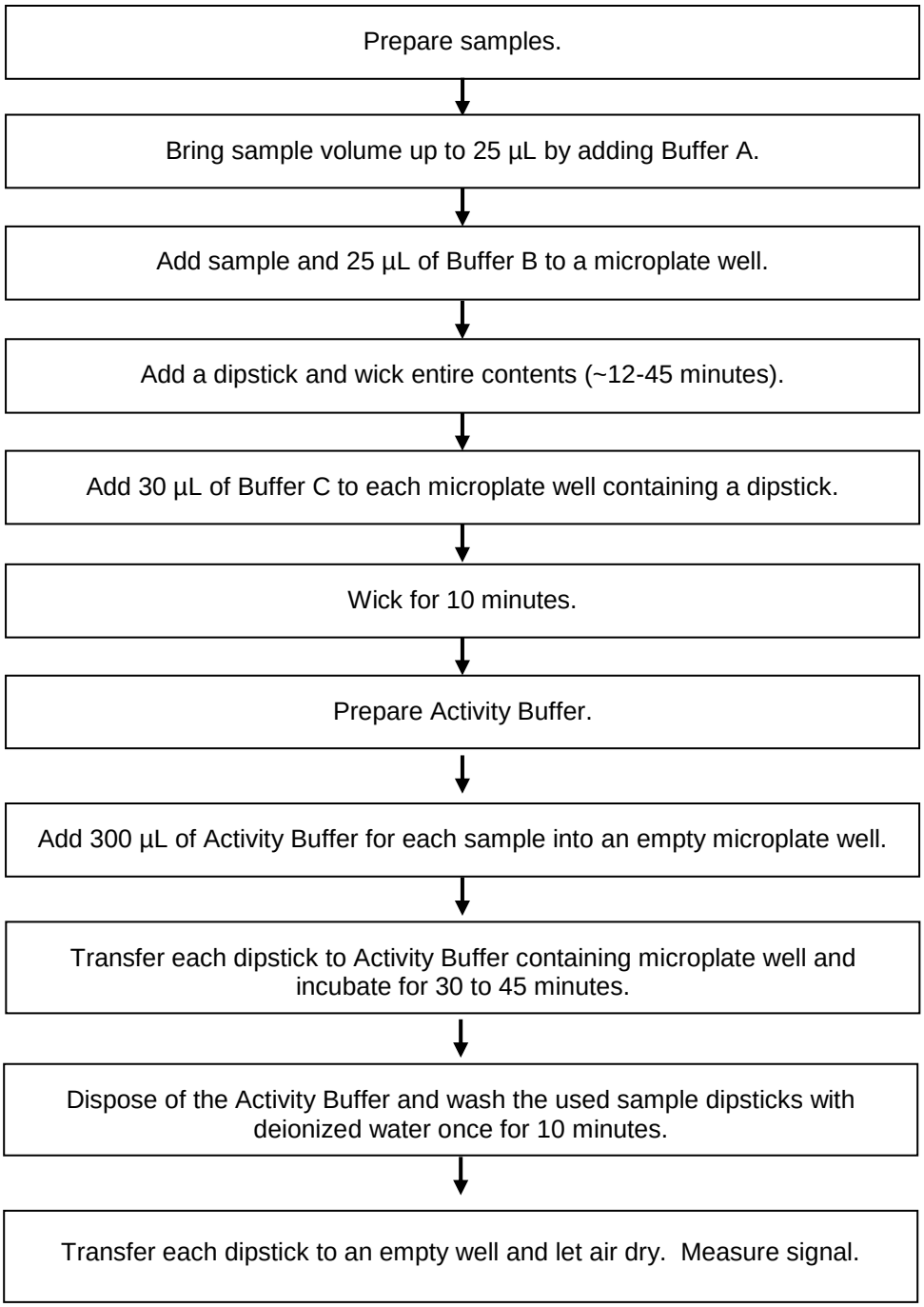
13. Add 300 µL deionized water to an empty well of the microplate.

14. Once the dipstick(s) are developed add them to the well with deionized water for 10 minutes

15. Dry the dipstick and measure the signal intensity with a dipstick reader (MitoSciences' MS-1000 Dipstick Reader) or other imaging system, e.g. flat-bed scanner.

FLOW CHART

For quick reference only. Be completely familiar with the previous details of this protocol before performing the assay.

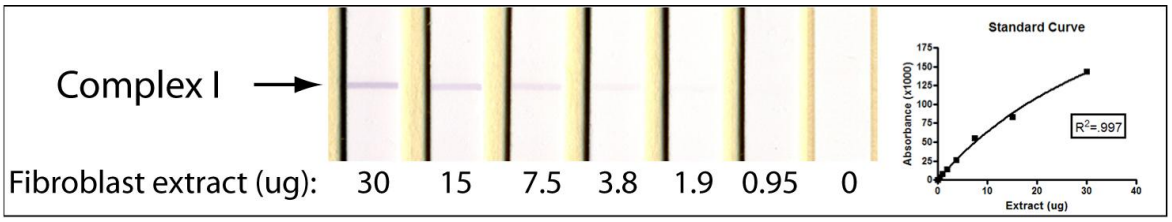


EXAMPLE EXPERIMENT

Below is an example using the MS130 kit to measure Complex I activity in human fibroblast samples. Samples were prepared as described in the Sample Preparation section. All data were analyzed using MitoSciences' MS1000 Dipstick Reader and GraphPad software.

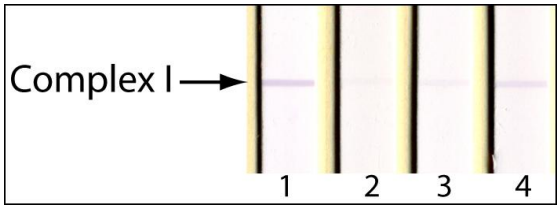
Step 1. Generating a standard curve

Shown are developed dipsticks from a 1:2 dilution series using a positive control sample and the associated standard curve. Starting material was 30 µg of fibroblast protein extract.



Step 2. Analysis of samples

Based on the standard curve, 15 µg of protein extract were loaded onto a dipstick for each sample. The figure below shows four developed dipsticks, a control sample (1) and three unknowns (2-4). The analysis of the signal intensity and interpolation from the standard curve showed that the unknown samples have between 14-61% of normal Complex I activity levels.



Control	Absorbance (x1000)	%normal
1	80.2	100
unknowns		
2	13.8	14.02
3	29.3	31.12
4	53.3	60.86

TROUBLESHOOTING GUIDE

Signal is saturated

It is very important that the amount of sample used is within the working range of the assay (use a best fit line for interpolation). Therefore, it is crucial to determine the working range for your sample type and avoid the region of signal saturation.

Signal is too weak

This occurs when the sample lacks measurable amounts of the protein. Increase the signal by adding more sample protein to another dipstick, or leave the dipstick in the activity solution for longer to maximize the signal

Sample is not wicking up the dipstick

If the dipstick is not handled gently, the nitrocellulose membrane and wicking pad may become separated. Check this junction and simply pinch the dipstick at this point to reconnect the two. Check for proper wicking of the sample.

Telephone: 1-800-910-6486 or 541-284-1800 · Fax: 541-284-1801
www.mitosciences.com
Customer Service: sales@mitosciences.com · Tech Support: support@mitosciences.com