

**ab110043 –
Fumarase Specific
Activity Microplate Assay
Kit**

Instructions for Use

For the quantitative measurement of Fumarase activity and quantity in Human and Bovine samples, activity only in Rat samples only

This product is for research use only and is not intended for diagnostic use.

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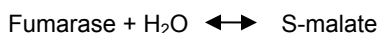
1. Introduction

Fumarase (FH, Fumarate hydratase, P07954) is a 50 kDa enzyme that catalyzes the reversible hydration/dehydration of fumarate to S-malate (EC 4.2.1.2) in the mitochondrial Krebs cycle, see below. A second fumarase isoform exists without the mitochondrial import sequence. This cytoplasmic form of the enzyme metabolizes cytoplasmic fumarate, a byproduct of the urea and amino acid metabolism.

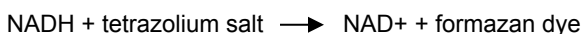
Defects in fumarase are the cause of fumarase deficiency [MIM 606812] which is characterized by progressive encephalopathy, developmental delay, hypotonia, cerebral atrophy and lactic/pyruvic academia. Additionally FH is a tumor suppressor gene; inactivating mutations in the FH gene are responsible for hereditary leiomyomataosis renal cell carcinoma (HLRCC) [MIM 605839]. It is likely that the inhibition of fumarase leads to an increase in fumarate which results in the inhibition of HIF prolyl hydroxylase (PHD). This leads to Hif1 α accumulation and transcriptional upregulation of HIF target genes such as PDK1 and GLUT1 resulting in a shift to a primarily glycolytic cellular metabolism with impairment of mitochondrial oxidative phosphorylation. As an example, in a study of a number of lymphoblastoid and fibroblast cell lines from individuals with HLRCC, harboring 16 different FH mutations, it was determined that fumarase activity was a valuable diagnostic in cases with undetected FH mutation. Defects in FH are also the cause of

multiple cutaneous and uterine leiomyomata (MCUL1) in which affected individuals develop benign smooth muscle tumors (leiomyomata) of the skin and uterus in females [MIM 150800].

This FH assay kit, ab110043, measures the following fumarase catalyzed reaction:



The generated malate is detected in a coupled reaction by the presence of malate dehydrogenase in the activity buffer:



(↑ Absorbance at 450 nm)

ab110043 (MS780) improves upon conventional fumarase activity assays in two ways. First, this assay immunocaptures in each well only fumarase; this removes all other enzymes of the Krebs cycle and NAD⁺/NADH utilizing enzymes. Second, after activity measurement, in the same well/s, the quantity of enzyme is measured by adding a fumarase specific antibody which is detected by a colorimetric label (HRP). This reaction takes place in a time dependant manner proportional to the amount of enzyme captured in each well. By combining activity and quantity measurements, the relative specific activity can be determined. Specific activity is useful for measuring up or down regulation of fumarase activity by site-specific mutation, modification or damage.

This assay has been validated for use with human tissue samples and many human cultured cell lines. Typical ranges for several sample types are described below. It is highly recommended to prepare multiple dilutions for each sample to ensure that each is in the working range of the assay (see Data Analysis section).

Table I: Typical working ranges for activity and quantity of samples in $\mu\text{g/ml}$ (Note however the assay requires 0.1 ml sample in each well):

Cultured Human whole cell extracts:	
• HeLa	30 μg - 500 $\mu\text{g/ml}$
• Fibroblasts	50 μg - 1000 $\mu\text{g/ml}$
• HepG2	50 μg - 500 $\mu\text{g/ml}$
• HL60	125 μg - 500 $\mu\text{g/ml}$
• Transformed B Lymphocytes	10 μg - 500 $\mu\text{g/ml}$
Tissue extracts (e.g. heart)	50 μg - 500 $\mu\text{g/ml}$
Tissue mitochondria (e.g. liver)	2.5 μg - 250 $\mu\text{g/ml}$
Bovine heart mitochondria	50 μg - 500 $\mu\text{g/ml}$
Rat Liver extracts (activity only)	15 μg - 125 $\mu\text{g/ml}$

2. Assay Summary

Prepare Sample (approximately 1 hour)

- Homogenize the sample and pellet (12000g, 20 min, 4°C)
- Resuspend the sample in 1X Extraction buffer
- Incubate on ice for 20 minutes.
- Centrifuge at 12000 x g for 20 minutes and then collect supernatant.
- Determine protein concentration and dilute to within recommendation range



Load plate (3 hours)

- Load sample(s) on the plate being sure to include a dilution series of a reference or normal sample and a buffer control as null reference.
- Incubate 3 hrs at room temperature.



Activity measurement (1 hour)

- Empty wells and wash each well twice with 300 μ l 1X Wash Buffer.
- Prepare activity solution
- Measure activity in a standard microplate reader for up to 10-60 mins OD₄₅₀



Detector antibody incubation (1 hour)

- Empty wells and wash each well twice with 300 μ l 1X Wash Buffer.
- Add 100 μ l of 1X Detector antibody to every well used.
- Incubate for 60 mins at room temperature.



HRP label incubation (1 hour)

- Empty wells and wash each well twice with 300 μ l 1X Wash Buffer.
- Add 100 μ l of 1X HRP Label to every well used.
- Incubate for 60 mins at room temperature.



Quantity measurement (15 min)

- Empty wells, wash each four times with 300 μ l 1X Wash Buffer.
- Add 100 μ l of Development Solution.
- Measure OD₆₀₀ at 20-second intervals for 15 minutes at room temperature.

3. Kit Contents

Sufficient materials are provided for 96 measurements in a microplate.

Part Number	Item	Quantity
8209730	200X Fumarate (1M)	0.25 ml
8201093	Extraction Buffer	15 ml
8209204	20X Wash Buffer	25 ml
8209731	200X β NAD ⁺	0.25 ml
8209732	2000X MDH	0.02 ml
8209702	100X Coupler	0.25 ml
8209701	100X Reagent Dye	0.25ml
8209203	10X Blocking Solution	10 ml
8209803	1X Development Solution	12 ml
8209733	1X Base Buffer	25 ml
8209734	100X Detector Antibody	0.125 ml
8209510	100X HRP Label	0.125ml
8209736	96-well microplate (12 strips)	1

4. Storage and Handling

All components are shipped cold. Upon receipt store 200X Fumarate, 200X β NAD⁺, 100X Coupler and 100X Reagent Dye at -20°C or preferably at -80°C for longer stability. Store all other components at 4°C.

5. Additional Materials Required

- Spectrophotometer that measures absorbance:
Activity: (450nm) Quantity: (450, 600 or 650 nm)
- Multichannel pipette (50 - 300 μ l) and tips
- 1.5 ml microtubes
- A fine needle
- Optional 1N HCl

6. Preparation of Buffers and Samples

Note: This protocol contains detailed steps for measuring FH activity in human samples. Be completely familiar with the protocol before beginning the assay. Do not deviate from the specified protocol steps or optimal results may not be obtained.

A. Buffer Preparation

Prepare 1X Wash Buffer by adding 25 ml 20X Wash Buffer to 475 ml deionized water.

Prepare 1X Incubation Buffer by adding 10 ml 10X Blocking Solution to 90 ml 1X Wash Buffer.

Before use (in Assay Method Step A4) prepare 1X Activity Solution. For an entire plate add 0.125 ml 200X β NAD⁺, 12.5 μ l MDH, 0.25 ml 100X Coupler, 0.25 ml Reagent dye to the 24.25 ml Base Buffer provided. Finally immediately prior to addition to the wells add 0.125 ml 200X Fumarate (final concentration 5 mM fumarate).

Before use (in Assay Method Step B2) prepare 1X Detector Antibody. For an entire plate add 0.125 ml 100X Detector Antibody to 12.375 ml 1X Incubation Buffer.

Before use (in Assay Method Step B4) prepare 1X HRP Label. For an entire plate add 0.125 ml 100X HRP Label to 12.375 ml 1X Incubation Buffer.

B Sample preparation

Note: Samples must be detergent extracted by the provided Extraction buffer. To do this cell pellets, tissue homogenate pellets, or mitochondrial pellets must be prepared.

1. Briefly homogenate and mitochondrial preparations are typically centrifuged at 12000 g for 20 min at 4°C to pellet. Cells are typically pelleted at 500 g for 10 min at 4°C. Estimate the volume of the pellet and then resuspend the sample pellet in 9 volumes of Sample Extraction Buffer.

A total of 15 ml 1X Extraction buffer is provided. Example - for analyses of multiple samples this is enough to extract 15 samples at 1 ml, 30 at 0.5 ml, 60 at 0.25 ml etc.

2. Incubate extracts on ice for 20 minutes. Centrifuge 12000 x g, 4°C, for 20 minutes. Save the supernatant and discard the pellet.
3. Determine the protein concentration of the supernatant extract (a protein assay method unaffected by the presence of detergent is critical e.g. BCA assay recommended or OD280).

4. The sample should be diluted to within the working range of the assay as described in Assay Method Step A1 and first consulting Table I. Undiluted extract can be frozen at -80°C .

7. Assay Method

A. Activity Measurement

1. Detergent extracted samples should be diluted in 1X Incubation Buffer. A dilution series of samples is recommended to ensure that samples are within the working range of the activity and the quantity assays. Also include a buffer control (1X Incubation Buffer only) as a null or background reference. Add 100 μl of each diluted sample into individual wells.
2. Cover/seal the plate and incubate for 3 hours at room temperature with shaking.
3. Wash the plate
 - a. Empty the wells by turning the plate over a receptacle and firmly shaking out the well contents in one rapid downward motion. Dispose of biological samples appropriately.

- b. Rapidly add 300 μ l 1X Wash Buffer to each well. The wells must not become dry during any step. Repeat this wash once more for a total of two washes. After the last wash strike the microplate surface onto paper towels to remove excess liquid.
4. Gently add 200 μ l 1X Activity Solution (step A3) to each well minimizing the production of bubbles.
5. Pop any bubbles immediately and record absorbance in the microplate reader prepared as follows:

Mode:	Kinetic
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Wavelength:	450 nm
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Time:	10 min – 1 hr (as desired)
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Interval:	20 sec -1 min
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Shaking:	Shake between readings
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Alternative– In place of a kinetic reading, at a user defined time/s, record the OD 450 nm in all wells. However in this case it is important the reaction is proceeding in all wells at a close to linear rates.

6. Record the data for analysis and proceed to section B – Quantity Measurement.

B. Quantity Measurement

1. Repeat the wash procedure in step A3. Collect and dispose of solutions and any added enzyme inhibitors appropriately.
2. Add 100 μ l of 1X Detector Antibody to each well used. Cover/seal the plate and incubate for 1 hour at room temperature with shaking.
3. Repeat the wash procedure in step A3.
4. Add 100 μ l of 1X HRP Label to each well used. Cover/seal the plate and incubate 1 hour at room temperature with shaking. Meanwhile, prepare the microplate spectrophotometer using the parameters described below (Step B6).
5. Repeat the wash procedure in step A3 however performing a total of four washes.

6. Add 100 μ l HRP Development Solution to each empty well, rapidly pop bubbles, and immediately record the blue color development in the microplate reader prepared as follows:

Mode:	Kinetic
Wavelength:	600 nm
Time:	up to 15 min
Interval:	20 sec -1 min
Shaking:	Shake between readings

Alternative– In place of a kinetic reading, at a user defined time, record the endpoint OD data at 600 nm or 450 nm. The reaction can be stopped by adding 50 μ l stop solution (1N HCl) to each well and record the OD at 450 nm - however an intense, sub-saturated, blue color could become saturating by stopping the reaction and measuring at 450nm. However in this case it is important the reaction is proceeding in all wells at close to a close to linear rates.

Record the data and proceed to Data Analysis.

8. Data Analysis

A. Calculation of activity and Quantity

Determine the relative activity and quantity of an unknown sample by first creating standard curves for the reference sample.

The following two-fold dilution series of HeLa cell extract was prepared in incubation buffer:

0, 8, 16, 32, 64, 125, 250, 500, 1000 $\mu\text{g/ml}$ of HeLa cell extract

Activity and quantity data were sequentially collected as described in this protocol using a microplate reader. Standard curves of these reference sample data were prepared using the microplate reader software, which is capable of a 4-parameter data analysis (Figure 1). Activity and quantity are clearly measurable in the 30-500 $\mu\text{g/ml}$ range when such a fit was applied (alternatively raw data can be exported to other graphing software).

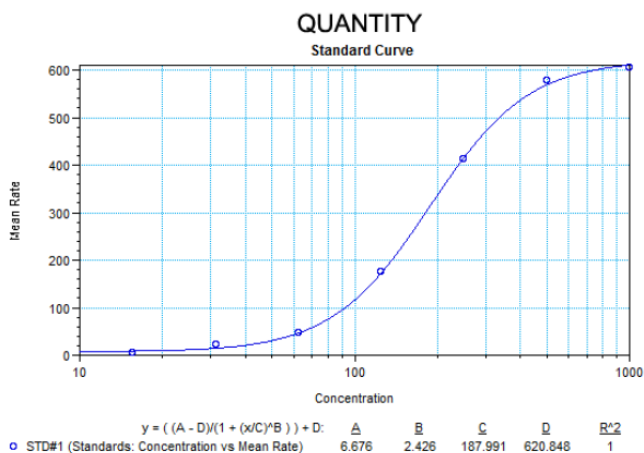
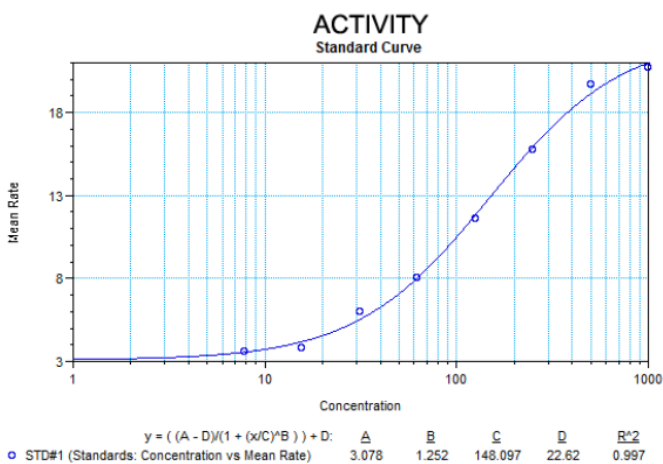


Figure 1. A reference cell line (example – HeLa) is used to construct standard curves for activity and quantity.

Unknown samples are interpolated from these reference sample graphs. By doing this for activity, the amount of reference sample required to generate the same amount of activity as the unknown sample is determined. This ratio (or %) of amount of unknown sample: reference sample is the relative activity.

Similarly this is performed to determine the relative quantity in an unknown sample. Together these numbers identify any alteration in the activity and or in the quantity of FH of an unknown sample.

Example dataset – Analysis of control and B lymphocytes samples.

A human transformed B lymphocyte cell line (P1) harboring a translocated chromosome and partial trisomy was reported to have elevated FH activity due to the additional FH gene. This cell line was compared to two unaffected control B lymphocyte cell lines (C1 and C2). At each sample dilution, in the normal working range, the activity and quantity of FH was confirmed to be higher in P1 than in C1 or C2 which were both similar (Figure 2).

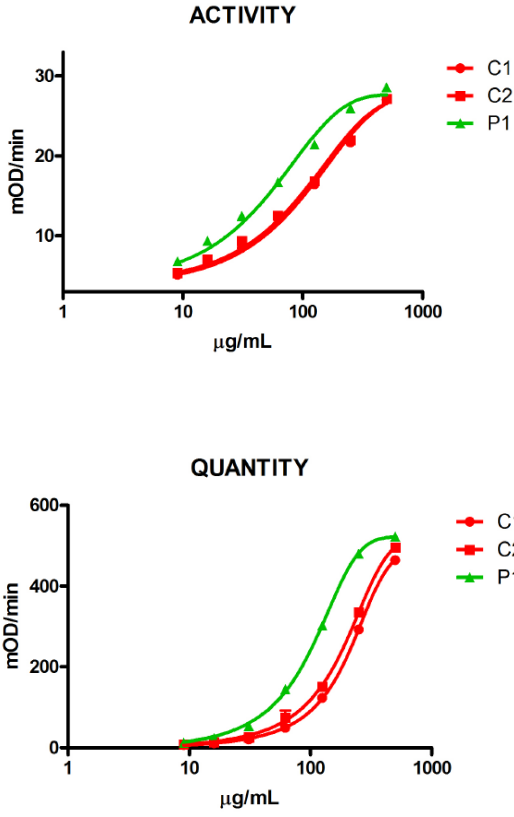


Figure 2. The sample P1 has extra genetic material including a copy of the FH gene. Analysis of this cell line alongside two control cell lines shows that P1 has a greater amount and activity of FH at all sample dilutions. However the specific activity (ratio of activity to quantity) of FH in the sample cell line is very similar to the control cell lines.

Control cell line C1 was designated as the reference cell line to create a relative standard curve to which all data are

compared. The ratio of the relative activity and quantity in all samples at all dilutions is very similar indicating that the measured specific activity of the enzyme in each sample and at all dilutions is the same (Figure 3).

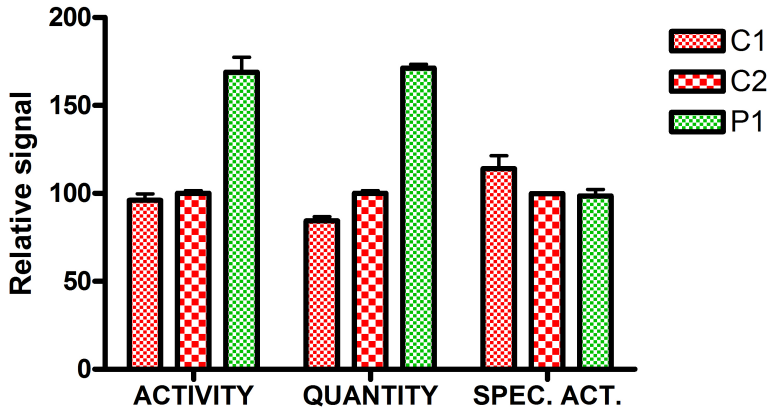


Figure 3. The activity is 68% higher in P1 than the control cell line; the quantity is 70% higher. Therefore since both signals increased by similar amounts, the relative specific activity is the same in P1 as the control cell lines, C1,C2.

B. Reproducibility

Typical intra-assay (well-well) variations (same sample) <7% (n=10).

Typical inter-assay (plate-plate) variation (same sample) <10% (n=10).

9. Specificity

This kit measures activity and amount of fumarase in human and bovine cells and tissues.

This kit does is not compatible with mouse samples, however the activity assay is compatible with rat samples.

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