FHIT Fluorometric Activity Assay Kit

Item No. 702510

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size	Storage Temperature
400676	FHIT Assay Buffer (5X)	1 vial/20 ml	4°C
400677	FHIT Substrate	1 vial/60 µl	-20°C
400678	TG Standard	1 vial/40 µl	-20°C
400679	FHIT Positive Control	1 vial/50 µl	-20°C
700416	DTT (1M) Assay Reagent	1 vial/1 ml	-20°C
400091	Half-Volume 96-Well Solid Plate (black)	1 plate	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

This kit may not perform as described if any reagent or procedure is replaced or modified.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section (see page 3) and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring fluorescence with excitation and emission wavelengths of 485-495 nm (λ_{max}: 491 nm) and 530-540 nm (λ_{max}: 535 nm), respectively, and incubating at 37°C
2. An orbital microplate shaker
3. Adjustable pipettes; multichannel or repeating pipettor recommended
4. A source of pure water; glass-distilled water is acceptable. *NOTE: UltraPure water is available for purchase from Cayman (Item No. 400000).*

INTRODUCTION

Background

Fragile histidine triad diadenosine triphosphatase (FHIT) is a tumor suppressor and a His-x-His-x-His motif-containing hydrolase.[1-3] It converts diadenosine triphosphate (Ap_3A), an alarmone that increases in response to cell stress, into AMP and ADP, and also hydrolyzes Ap_4A and ATP.[1,4,5] FHIT is expressed in the spleen, brain, kidney, lung, and liver and localizes to the plasma membrane, nucleus, mitochondria, and cytoplasm.[6-8] It is involved in tumor suppression and genome stability.[2] Overexpression of *FHIT* induces apoptosis in lung cancer cells and reduces tumor growth in lung cancer mouse xenograft models.[9] Homozygous or heterozygous loss of *FHIT* expression is prevalent in several cancers, and the *FHIT* gene contains a common fragile site, *FRA3B*, which is a translocation breakpoint associated with hereditary kidney cancer.[3] Increased methylation of the *FHIT* promoter and decreased protein levels of FHIT in active inflammatory colon tissues are associated with Crohn's disease.[10] FHIT levels in the lungs are decreased in patients with pulmonary arterial hypertension.[11] The continued study of FHIT and its activity is important for determining its mechanistic role in cancer and other diseases.[3]

About This Assay

Cayman's FHIT Fluorometric Activity Assay Kit provides a convenient method of detecting FHIT activity in cell and tissue lysates. Measurement of the FHIT activity is carried out by monitoring the hydrolysis of an FHIT substrate analog, TG-1AdaAMP, generating free Tokyo Green™, whose fluorescence can be easily monitored at excitation and emission wavelengths of 485 and 535 nm, respectively (see Figure 1).[4] The rate of increase in fluorescence is directly proportional to the levels of active FHIT in the sample.

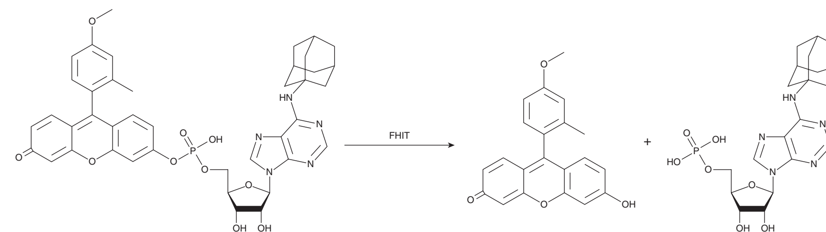

Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. **DTT (1M) Assay Reagent – (Item No. 700416)**

   This vial contains 1 ml of DTT (1M) Assay Reagent, which is ready to use as supplied. If not using all at once, prepare aliquots and store at -20°C. Avoid multiple freeze-thaw cycles.

2. **FHIT Assay Buffer (5X) – (Item No. 400676)**

   This vial contains 20 ml of concentrated FHIT Assay Buffer. To prepare the assay buffer, mix 5 ml of FHIT Assay Buffer (5X) with 20 ml of pure water and add 50 µl of DTT (1 M) Assay Reagent. The complete assay buffer should be used in the assay and for dilution of the substrate, standard, and positive control. The assay buffer should be prepared just prior to use and kept on ice where it will be stable for four hours.

3. **FHIT Substrate – (Item No. 400677)**

   This vial contains 60 µl of an FHIT substrate analog, TG-1AdaAMP. If not using all at once, prepare aliquots and store at -20°C. Avoid multiple freeze-thaw cycles and keep protected from light.

   Dilute the substrate 20-fold by mixing 50 µl of FHIT Substrate with 950 µl of assay buffer. This is a sufficient volume of substrate to assay 100 wells. Scale as needed. This solution will be stable for one hour at room temperature.

4. **TG Standard - (Item No. 400678)**

   This vial contains 40 µl of 1 mM Tokyo Green™ (TG) in DMSO. If not using the standard all at once, prepare aliquots and store at -20°C. Avoid multiple freeze-thaw cycles and protect from light.

   Prior to the assay, dilute the TG Standard to 16 µM by adding 16 µl of TG Standard to 984 µl of assay buffer. Mix thoroughly.

5. **FHIT Positive Control - (Item No. 400679)**

   This vial contains 50 µl of recombinant human FHIT enzyme in 50% glycerol, which can be used as a positive control. If not using all at once, prepare aliquots and store at -20°C. **Do not store at -80°C. Do not vortex.**

   Immediately prior to use in the assay, dilute 10 µl of the positive control with 40 µl of assay buffer and place on ice where it will be stable for one hour.

Sample Preparation

NOTE: To determine FHIT specific activity, it will be necessary to determine the protein concentration of the samples being tested. Protein determination kits are available for purchase from Cayman (Item Nos. 701780 | 760200).

Tissue Homogenate

1. Prior to dissection, rinse the tissue with PBS, pH 7.4, to remove any red blood cells and clots.
2. Homogenize 100-300 mg tissue in 0.5 to 1 ml of ice-cold assay buffer.
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Transfer the supernatant to a clean tube and store on ice. If not assaying on the same day, store the sample at -80°C.

Cell Lysate

1. Collect cells (~5 x 10^6) by centrifugation (*i.e.*, 1,000-2,000 x g for 10 minutes at 4°C). For adherent cells, use a rubber cell scraper instead of proteolytic enzymes.
2. Homogenize the cell pellet in 0.5-1 ml ice-cold assay buffer.
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Transfer the supernatant to a clean tube and store on ice. If not assaying on the same day, store the sample at -80°C.

ASSAY PROTOCOL

Standard Curve Preparation

Label six clean glass or polystyrene test tubes A-F. Add the amount of TG standard solutions and assay buffer to each tube as described in Table 1, below. The diluted standards will be stable for four hours at room temperature in the dark.

Tube	Assay Buffer (µl)	Volume of TG Standard (µl)	TG Standard (pmoles in well)
A	0 µl	200 µl of diluted TG Standard (16 µM)	800 pmol
B	200 µl	200 µl of diluted TG Standard (16 µM)	400 pmol
C	200 µl	200 µl of TG Standard B	200 pmol
D	200 µl	200 µl of TG Standard C	100 pmol
E	200 µl	200 µl of TG Standard D	50 pmol
F	200 µl	0 µl	0 pmol

Table 1. Preparation of the TG standards

Plate Set Up

There is no specific pattern for using the wells on the plate. It is recommended that two wells be designated for the FHIT Positive Control. It is suggested that each sample be assayed in duplicate and that the contents of each well are recorded on the template sheet provided on page 25. A typical layout of samples to be measured in duplicate is provided below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	1	1	9	9	17	17	25	25	33	33
B	B	B	2	2	10	10	18	18	26	26	34	34
C	C	C	3	3	11	11	19	19	27	27	35	35
D	D	D	4	4	12	12	20	20	28	28	36	36
E	E	E	5	5	13	13	21	21	29	29	37	37
F	F	F	6	6	14	14	22	22	30	30	38	38
G	PC	PC	7	7	15	15	23	23	31	31	39	39
H	Bkg	Bkg	8	8	16	16	24	24	32	32	40	40

A-F = Standard Wells
PC = Positive Control Wells
Bkg = Background Wells
1-40 = Sample Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 50 µl in all of the wells.
- Use freshly prepared assay buffer in the assay.
- All reagents should be prepared as described above. The FHIT Positive Control and assay buffer should be kept on ice and all other reagents should be kept at room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- 24 samples can be assayed in triplicate or 40 in duplicate.
- It is recommended for samples to be assayed at several dilutions to ensure accurate measurement of activity and that samples fall within the linear range of the assay.
- The assay is performed at 37°C.
- Monitor the fluorescence with excitation and emission wavelengths of 485 and 535 nm, respectively.

Performing the Assay

1. Add the appropriate amount of reagents to the designated wells according to the table below.

Reagent	Standard Wells	Positive Control Wells	Sample Wells	Background Wells
Assay Buffer	-	30 µl	30 µl	40 µl
TG Standards	50 µl	--	--	--
Diluted FHIT Positive Control	--	10 µl	--	--
Sample	--	--	10 µl	--

Table 2. Pipetting summary

2. Quickly initiate the reactions by adding 10 µl of the diluted FHIT Substrate to all of the wells except the Standard Wells.
3. Mix thoroughly by gently pipetting up and down four to six times. Avoid forming bubbles. Thorough mixing is absolutely critical to the performance of the assay.
4. Immediately measure the fluorescence intensity with excitation and emission wavelengths of 485 and 535 nm, respectively, once every two minutes for 30 minutes at 37°C. If FHIT activity is low, continue reading for up to 60 minutes.

ANALYSIS

Calculations

Prepare the Standard Curve:

1. Determine the average endpoint fluorescence of each standard.
2. Subtract the average fluorescence of standard F from itself and all other standards to obtain corrected standard measurements (CSM).
3. Plot the CSM values of each standard as a function of the final amount of the standard in pmol from Table 1 on page 11. See Figure 3, below, for a typical standard curve. The slope of the standard curve (RFU/pmol) will be used to calculate the rate in pmol/min.

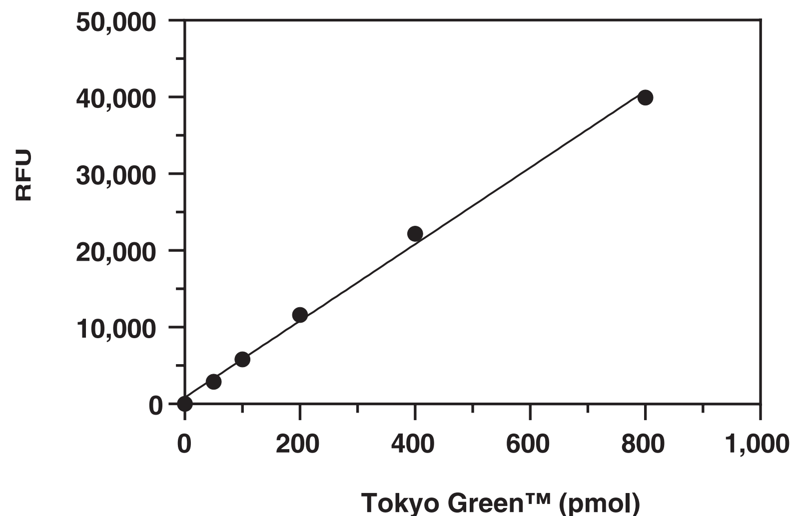

Figure 3. Typical standard curve

Determine the FHIT Activity in Sample and Positive Control Wells:

1. Determine the change in fluorescence per minute (RFU/min) for each sample, positive control, and background by plotting the fluorescence values as a function of time to obtain the slope (rate) of the linear portion of the curve (a graph is shown using various samples, see Figure 4, page 17).
2. If the average background rate is >0, subtract this value from the average rates of each sample and the positive control.
3. Calculate the FHIT activity (U/ml) using the equation below. One unit is defined as 1 nmol of substrate hydrolyzed per minute under the specified conditions of this assay. Use the following formula to calculate the FHIT activity:

$$\text{FHIT activity (U/ml)} = \left[\frac{\text{rate (RFU/min)}}{\text{standard curve slope (RFU/pmol)}}\right] \text{X} \ \frac{\text{dilution factor}}{10\ \mu\text{l}}$$

4. **Optional:** If the protein concentration of the samples has been determined, specific activity can be calculated using the equation below.

$$\text{specific activity} \left(\frac{\text{U}}{\text{mg}}\right) = \frac{\text{activity (U/ml)}}{\text{sample protein concentration (mg/ml)}}$$

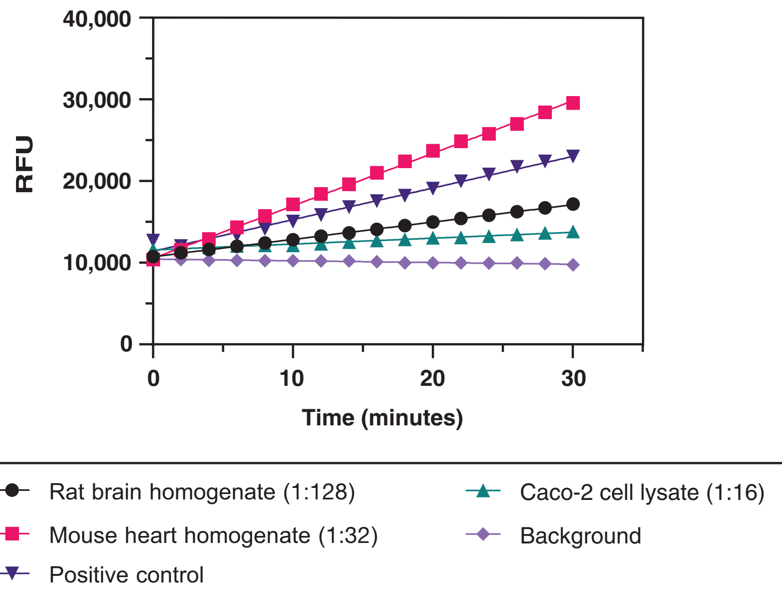

Figure 4. Activity of the FHIT Positive Control and various samples

Performance Characteristics

Sensitivity:

The limit of detection for this assay is 0.06 U/ml.

Precision:

When a series of 24 FHIT activity measurements were performed on the same day under the same experimental conditions, the intra-assay coefficients of variation were 4 and 3% in Caco-2 cell lysate and rat brain homogenate, respectively. When a series of sample measurements were performed on six different days under the same experimental conditions, the inter-assay coefficients of variation were 4 and 8%, in Caco-2 cell lysate and rat brain homogenate, respectively.

Z' Factor:

Z' Factor is a term used to describe the robustness of an assay, which is calculated using the equation below.[12]

$$Z' = 1 - \frac{3\sigma_{c+} + 3\sigma_{c-}}{|\mu_{c+} - \mu_{c-}|}$$

Where σ: Standard deviation
μ: Mean
c+: Positive control
c-: Negative control

The theoretical upper limit for the Z' factor is 1.0. A robust assay has a Z' factor >0.5. The Z' factor for Cayman's FHIT Fluorometric Activity Assay Kit was determined to be 0.87.

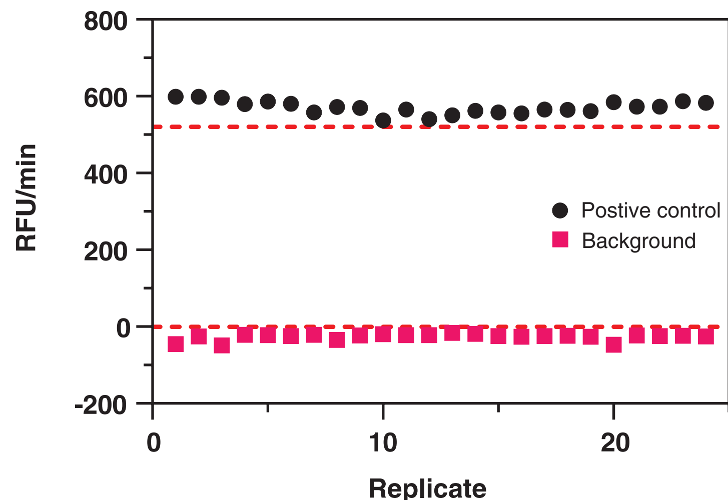

Figure 5. Typical performance data for the FHIT Fluorometric Activity Assay Kit. Data are shown from 24 replicates each for background and positive control prepared as described in the kit booklet. The calculated Z′ factor for this experiment was 0.87. The dashed lines correspond to three standard deviations from the mean for each control value.

Interferences

The following reagents were tested for interference in the assay.

Reagent		Will Interfere
Buffers	M-PER	Yes
	RIPA	Yes
	PBS	Yes
	T-PER	No
	HEPES (50 mM, pH 7.2-7.4)	No
	Tris (50 mM, pH 7.2-7.4)	No
Detergents	Triton-100 (>0.0025%)	Yes
	Polysorbate 20 (>0.025%)	Yes
	$C_{12}E_8$ (>0.025%)	Yes
Protease Inhibitors/ Enzymes	Mini protease inhibitor tablet, EDTA-free (1 tablet/10 ml)	No
Solvents	DMSO (>0.5%)	Yes

Table 3. Interferences

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of replicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No activity detected in samples and positive control	Substrate was not added to the wells	Make sure to add all the components to the wells and re-assay
No signal increase over time observed in the samples	A. FHIT activity is too low to detect B. No FHIT in the sample C. If the RFU at t_0 is much higher than the background RFU, the reaction has already reached completion; the enzyme level is too high	A. Concentrate the sample using a concentrator with a MW cut-off of 3 kDa and re-assay B. Verify expression and re-assay C. Dilute the sample and re-assay
No signal change over time in positive control wells	A. Positive control is inactive B. Positive control was not added to the well	A. Prepare a fresh positive control and verify proper storage and handling B. Make sure to add all the components to the wells and re-assay

Item No.	Reagent	Procedure
400676	FHIT Assay Buffer (5X)	Dilute to 1X and add DTT (1M) Assay Reagent to a final concentration of 2 mM.
400677	TG Standard	Dilute 16 µl with 984 µl of assay buffer. See page 11 for the preparation of standards.
400678	FHIT Substrate	Dilute the FHIT Substrate 1:20 with assay buffer.
400679	FHIT Positive Control	Dilute the FHIT Positive Control 1:5 with assay buffer.

Table 4. Reagent preparation summary

Procedure	Standard Wells	Positive Control Wells	Sample Wells	Background Wells
Add TG Standards	50 µl	--	--	--
Add assay buffer	--	30 µl	30 µl	40 µl
Add diluted FHIT Positive Control	--	10 µl	--	--
Add sample	--	--	10 µl	--
Add diluted FHIT Substrate	--	10 µl	10 µl	10 µl
Mix by pipetting up and down four to six times. Read fluorescence at excitation and emission wavelengths of 485 nm and 535 nm, respectively, every two minutes for 30 minutes at 37°C.				

Table 5. Assay summary

References

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NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.

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