FSP1 Fluorescent Inhibitor Screening Assay Kit

Item No. 701900

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Customer Service 800.364.9897
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1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -80°C kit. After opening the kit, store individual components as stated below.

Item Number	Item	Quantity/Size	Storage
701901	FSP1 Assay Buffer (10x)	1 vial/5 ml	-20°C
701902	FSP1 Enzyme	1 vial/25 µl	-80°C
701903	FSP1 NADH	2 vials	-20°C
701904	FSP1 Resazurin	2 vials/2 ml	-20°C
701905	FSP1 Inhibitor (iFSP1)	1 vial/100 µl	-20°C
700001	DMSO Assay Reagent	1 vial/1 ml	RT
400091/ 400093	Half-Volume 96-Well Solid Plate (black) OR 384-Well Solid Plate (low volume; black)	1 plate	RT
400023	Foil Plate Cover	1 ea	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

Fax: 734-971-3640

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 with the ability to measure fluorescence with excitation and emission wavelengths of 540 and 590 nm, respectively
2. Adjustable pipettes; multichannel or repeating pipettor recommended
3. An orbital microplate shaker
4. A source of ultrapure water is recommended. Pure water - glass-distilled or deionized - may not be acceptable. *Note: UltraPure Water is available for purchase from Cayman (Item No: 400000).*
5. Microcentrifuge tubes

INTRODUCTION

Background

Ferroptosis is a type of non-apoptotic programmed cell death triggered by the iron-dependent accumulation of oxidized extra-mitochondrial lipid membrane polyunsaturated fatty acids (PUFAs).[1,2] It is triggered by small molecules or conditions that inhibit glutathione peroxidase (GPX4), an enzyme that reduces lipid peroxides to non-reactive lipid alcohols, or glutathione (GSH) biosynthesis.[3] Ferroptosis can be negatively regulated by iron chelators and enzymes that mediate intracellular antioxidant responses, such as GTP cyclohydrolase-1 (GCH1) and ferroptosis suppressor protein 1 (FSP1). FSP1, also known as apoptosis-inducing factor mitochondria-associated 2 (AIFM2), is a flavoprotein and NAD(P)H-dependent oxidoreductase that inhibits ferroptosis in a GPX4- and GSH-independent manner.[4-6] FSP1 localizes to the plasma membrane in a myristoylation-dependent manner, where it catalyzes the regeneration of the reduced form of coenzyme Q_{10} (CoQ_{10}), $CoQ_{10}H_2$, which functions as a radical-trapping antioxidant that inhibits lipid peroxidation. Expression of *AIFM2*, the gene encoding FSP1, positively correlates with resistance to ferroptosis inducers, and inhibition of FSP1 sensitizes cancer cells to RSL3-induced ferroptosis implicating FSP1 as a target for anticancer therapies.[4]

About This Assay

Cayman's FSP1 Fluorescent Inhibitor Screening Assay Kit provides a robust and easy-to-use platform for identifying novel inhibitors of human FSP1, a negative regulator of the ferroptosis pathway. The assay measures the reduction of resazurin by FSP1 into resorufin, which can be easily quantified using a fluorescence plate reader at excitation and emission wavelengths of 540 and 590 nm, respectively. The potent and reversible FSP1 inhibitor iFSP1 is included as a positive control.

PRE-ASSAY PREPARATION FOR 96-WELL ASSAY

Sample Preparation

All inhibitors, be they small molecules, natural products, or proteins, should be prepared in diluted FSP1 Assay Buffer at a concentration 20X the desired final assay concentration (*e.g.*, for 10 µM final assay concentration, a 200 µM stock should be made). This solution may contain up to 100% DMSO, 20% dimethyl formamide (DMF), or 20% short-chain alcohols (*e.g.*, MeOH, EtOH). The final concentration of organic solvents in the assay will then be ≤5% DMSO or ≤1% DMF, EtOH, or MeOH (see 'Effects of Solvents' on page 21).

Reagent Preparation

1. **FSP1 Assay Buffer (1X)**

   Mix 3 ml of FSP1 Assay Buffer (10X) (Item No. 701901) with 27 ml of water to make 30 ml of FSP1 Assay Buffer (1X). The FSP1 Assay Buffer (1X) should be discarded if not used within the same day. Once thawed, the FSP1 Assay Buffer (10X) may be stored at 4°C for at least one month.

2. **FSP1 Resazurin**

   Each vial contains 2 ml of Resazurin (Item No. 701904). It will be stable at room temperature for at least 2 hours.

3. **FSP1 NADH**

   Each vial contains lyophilized NADH (Item No. 701903). Reconstitute the contents of each vial with 2 ml Assay Buffer (1X). Reconstituted NADH will be stable at room temperature for at least 2 hours.

4. **FSP1 Enzyme (human, recombinant)**

   FSP1 Enzyme (Item No. 701902) should be thawed on ice and mixed prior to dilution. To dilute the enzyme, mix 21.6 µl of FSP1 Enzyme (human, recombinant) with 1,978.5 µl FSP1 Assay Buffer (1X). It is recommended that the enzyme be diluted immediately prior to performing the assay. The diluted enzyme should be stored on ice and used within 2 hours. The undiluted enzyme can be stored at -80°C, limiting freeze-thaw cycles.

5. **FSP1 Inhibitor (iFSP1)**

   This vial contains 100 µl of FSP1 Inhibitor (iFSP1) (Item No. 701905) in DMSO, which can be used as a positive control. If all of the FSP1 Inhibitor (iFSP1) will not be used at one time, aliquot the undiluted inhibitor and store at -20°C.

PRE-ASSAY PREPARATION FOR 384-WELL ASSAY

Sample Preparation

All inhibitors, be they small molecules, natural products, or proteins, should be prepared in diluted FSP1 Assay Buffer at a concentration 20X the desired final assay concentration (*e.g.*, for 10 µM final assay concentration, a 50 µM stock should be made). This solution may contain up to 25% DMSO, 5% dimethyl formamide (DMF), or 5% short-chain alcohols (*e.g.*, MeOH, EtOH). The final concentration of organic solvents in the assay will then be ≤5% DMSO or ≤1% DMF, EtOH, or MeOH (see 'Effects of Solvents' on page 21).

Reagent Preparation

1. **FSP1 Assay Buffer (1X)**

   Mix 3 ml of FSP1 Assay Buffer (10X) (Item No. 701901) with 27 ml of water to make 30 ml of FSP1 Assay Buffer (1X). The FSP1 Assay Buffer (1X) should be discarded if not used within the same day. Once thawed, the FSP1 Assay Buffer (10X) may be stored at 4°C for at least one month.

2. **FSP1 Resazurin**

   Each vial contains 2 ml of Resazurin (Item No. 701904). Dilute this solution 1:2 by adding 2 ml of Assay Buffer (1X). It will be stable at room temperature for at least 2 hours.

3. **FSP1 NADH**

   Each vial contains lyophilized NADH (Item No. 701903). Reconstitute the contents of each vial with 4 ml Assay Buffer (1X). Reconstituted NADH will be stable at room temperature for at least 2 hours.

4. **FSP1 Enzyme (human, recombinant)**

   FSP1 Enzyme (Item No. 701902) should be thawed on ice and mixed prior to dilution. To dilute the enzyme, mix 13.5 µl of FSP1 Enzyme (human, recombinant) with 2,486.5 µl FSP1 Assay Buffer (1X). It is recommended that the enzyme be diluted immediately prior to performing the assay. The diluted enzyme should be stored on ice and used within 2 hours. The undiluted enzyme can be stored at -80°C, limiting freeze-thaw cycles.

5. **FSP1 Inhibitor (iFSP1)**

   This vial contains 100 µl of FSP1 Inhibitor (iFSP1) (Item No. 701905) in DMSO. Dilute 4-fold by mixing 25 µl of FSP1 Inhibitor (iFSP1) with 75 µl of Assay Buffer (1X) - this can be used as a positive control. If all of the FSP1 Inhibitor (iFSP1) will not be used at one time, aliquot the undiluted inhibitor and store at -20°C.

ASSAY PROTOCOL FOR 96-WELL ASSAY

Plate Set Up

The 96-well plate(s) included with this kit is supplied ready to use. There is no specific pattern for using the wells on the plate. However, it is necessary to have three wells designated as 100% initial activity and three wells designated as background. It is suggested that each inhibitor, including the positive control FSP1 Inhibitor (iFSP1), be assayed in triplicate. A typical layout of samples to be measured in triplicate is shown in Figure 1, below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BW	BW	BW	6	6	6	14	14	14	22	22	22
B	A	A	A	7	7	7	15	15	15	23	23	23
C	P	P	P	8	8	8	16	16	16	24	24	24
D	1	1	1	9	9	9	17	17	17	25	25	25
E	2	2	2	10	10	10	18	18	18	26	26	26
F	3	3	3	11	11	11	19	19	19	27	27	27
G	4	4	4	12	12	12	20	20	20	28	28	28
H	5	5	5	13	13	13	21	21	21	29	29	29

BW - Background Wells
A - 100% Initial Activity Wells
P - Positive Control Wells
1-29 - Inhibitor Wells

Figure 1. Sample 96-well 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 100 µl in all the wells.
- Use the diluted assay buffer in the assay.
- All reagents should be prepared as described above and kept at room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- If the appropriate inhibitor concentration is not known, it may be necessary to assay at several concentrations.
- It is recommended to assay the samples in triplicate, but it is the user's discretion to do so.
- The assay is performed at room temperature.
- Monitor the fluorescence with an excitation wavelength of 540 nm and an emission wavelength of 590 nm.

Performing the Assay

1. **Background Wells**: add 75 µl FSP1 Assay Buffer (1X) and 5 µl of solvent (the same solvent concentration used to dissolve the unknown inhibitor and the positive control, FSP1 inhibitor (iFSP1)), to three wells. If different solvents are to be assayed at the same time, separate sets of background wells should be run for each solvent.
2. **100% Initial Activity Wells**: add 65 µl FSP1 Assay Buffer (1X), 10 µl of diluted FSP1 Enzyme, and 5 µl of solvent to three wells. If inhibitors in different solvents are to be assayed at the same time, separate sets of 100% initial activity wells should be run for each solvent.
3. **Inhibitor/Positive Control Wells**: add 65 µl FSP1 Assay Buffer (1X), add 10 µl of FSP1 Enzyme, and 5 µl of unknown inhibitor or the positive control, FSP1 Inhibitor (iFSP1), to three wells.
4. Mix the contents of the wells by pipetting gently.
5. Cover the plate with the Foil Plate Cover (Item No. 400023) and incubate for 10 minutes at room temperature.
6. Remove the plate cover and add 10 µl of resazurin to all wells being used.
7. Initiate the reaction by adding 10 µl of reconstituted NADH to all wells being used and mix by shaking the plate for 20 seconds on an orbital plate shaker.
8. Cover the plate and incubate for 15 minutes.
9. Remove the plate cover and read the plate with an excitation wavelength of 540 nm and an emission wavelength of 590 nm. It may be necessary to adjust the gain setting to allow for the measurement of all samples.*

*If desired, the assay may be read kinetically rather than as an endpoint. Reading the assay kinetically may increase the signal-to-background ratio. The fluorescence should be measured at least once per minute at room temperature for 15 minutes. Determine the initial rate based on the linear portion of the kinetic curve. Calculations can be performed as shown below substituting initial rates for average fluorescence.

ASSAY PROTOCOL FOR 384-WELL ASSAY

Plate Set Up

The 384-well plate(s) included with this kit is supplied ready to use. There is no specific pattern for using the wells on the plate. However, it is necessary to have three wells designated as 100% initial activity and three wells designated as background. It is suggested that each inhibitor, including the positive control FSP1 Inhibitor (iFSP1), be assayed in triplicate. A typical layout of samples to be measured in triplicate is shown in Figure 2, below.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	BW	BW	BW	14	14	14	30	30	30	46	46	46	62	62	62	78	78	78	94	94	94	110	110	110
B	A	A	A	15	15	15	31	31	31	47	47	47	63	63	63	79	79	79	95	95	95	111	111	111
C	P	P	P	16	16	16	32	32	32	48	48	48	64	64	64	80	80	80	96	96	96	112	112	112
D	1	1	1	17	17	17	33	33	33	49	49	49	65	65	65	81	81	81	97	97	97	113	113	113
E	2	2	2	18	18	18	34	34	34	50	50	50	66	66	66	82	82	82	98	98	98	114	114	114
F	3	3	3	19	19	19	35	35	35	51	51	51	67	67	67	83	83	83	99	99	99	115	115	115
G	4	4	4	20	20	20	36	36	36	52	52	52	68	68	68	84	84	84	100	100	100	116	116	116
H	5	5	5	21	21	21	37	37	37	53	53	53	69	69	69	85	85	85	101	101	101	117	117	117
I	6	6	6	22	22	22	38	38	38	54	54	54	70	70	70	86	86	86	102	102	102	118	118	118
J	7	7	7	23	23	23	39	39	39	55	55	55	71	71	71	87	87	87	103	103	103	119	119	119
K	8	8	8	24	24	24	40	40	40	56	56	56	72	72	72	88	88	88	104	104	104	120	120	120
L	9	9	9	25	25	25	41	41	41	57	57	57	73	73	73	89	89	89	105	105	105	121	121	121
M	10	10	10	26	26	26	42	42	42	58	58	58	74	74	74	90	90	90	106	106	106	122	122	122
N	11	11	11	27	27	27	43	43	43	59	59	59	75	75	75	91	91	91	107	107	107	123	123	123
O	12	12	12	28	28	28	44	44	44	60	60	60	76	76	76	92	92	92	108	108	108	124	124	124
P	13	13	13	29	29	29	45	45	45	61	61	61	77	77	77	93	93	93	109	109	109	125	125	125

BW - Background Wells
A - 100% Initial Activity Wells
P - Positive Control Wells
1-125 - Inhibitor Wells

Figure 2. Sample 384-well 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 25 µl in all the wells.
- Use the diluted assay buffer in the assay.
- All reagents should be prepared as described above and kept at room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- If the appropriate inhibitor concentration is not known, it may be necessary to assay at several concentrations.
- It is recommended to assay the samples in triplicate, but it is the user's discretion to do so.
- The assay is performed at room temperature.
- Monitor the fluorescence with an excitation wavelength of 540 nm and an emission wavelength of 590 nm.

Performing the Assay

1. **Background Wells**: add 10 µl FSP1 Assay Buffer (1X) and 5 µl of solvent (the same solvent concentration used to dissolve the unknown inhibitor and the positive control, FSP1 inhibitor (iFSP1)), to three wells. If different solvents are to be assayed at the same time, separate sets of background wells should be run for each solvent.
2. **100% Initial Activity Wells**: add 5 µl FSP1 Assay Buffer (1X), 5 µl of diluted FSP1 Enzyme, and 5 µl of solvent to three wells. If inhibitors in different solvents are to be assayed at the same time, separate sets of 100% initial activity wells should be run for each solvent.
3. **Inhibitor/Positive Control Wells**: add 5 µl FSP1 Assay Buffer (1X), add 5 µl of FSP1 Enzyme, and 5 µl of unknown inhibitor or the positive control, FSP1 Inhibitor (iFSP1), to three wells.
4. Mix the contents of the wells by pipetting gently.
5. Cover the plate with the Foil Plate Cover (Item No. 400023) and incubate for 10 minutes at room temperature.
6. Remove the plate cover and add 5 µl of resazurin to all wells being used.
7. Initiate the reaction by adding 5 µl of reconstituted NADH to all wells being used and mix by shaking the plate for 20 seconds on an orbital plate shaker.
8. Cover the plate and incubate for 15 minutes.
9. Remove the plate cover and read the plate with an excitation wavelength of 540 nm and an emission wavelength of 590 nm. It may be necessary to adjust the gain setting to allow for the measurement of all samples.*

*If desired, the assay may be read kinetically rather than as an endpoint. Reading the assay kinetically may increase the signal-to-background ratio. The fluorescence should be measured at least once per minute at room temperature for 15 minutes. Determine the initial rate based on the linear portion of the kinetic curve. Calculations can be performed as shown below substituting initial rates for average fluorescence.

ANALYSIS

Calculations

1. Determine the average fluorescence (AF) of each sample.
2. Subtract the AF of the background wells from the AF of the 100% initial activity and inhibitor wells. These are the corrected values.
3. Determine the percent inhibition or percent activity for each inhibitor using one of the following equations:

$$\% \text{ Inhibition} = \left[\frac{(\text{corrected 100\% initial activity} - \text{corrected inhibitor activity})}{\text{corrected 100\% initial activity}} \right] \times 100$$

$$\% \text{ Activity} = \left[\frac{(\text{corrected inhibitor activity})}{\text{corrected 100\% initial activity}} \right] \times 100$$

4. Graph the percent inhibition or percent activity as a function of inhibitor concentration to determine the IC_{50} value (the concentration at which there is 50% inhibition) of the inhibitor. Inhibition of recombinant human FSP1 by FSP1 Inhibitor (iFSP1) is shown in figure 3 (see page 19).

Performance Characteristics

Z´ Factor:

Z´ factor is a term used to describe the robustness of an assay, which is calculated using the equation below.[7]

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

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 FSP1 Fluorescent Inhibitor Screening Assay Kit was determined to be 0.89.

Sample Data:

The data shown here is an example of the data typically produced with this kit; however, your results will not be identical to these.

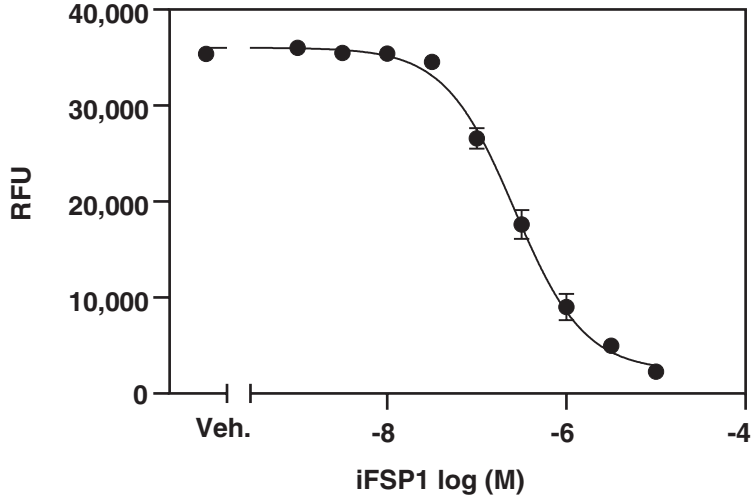

Figure 3. Inhibition of recombinant human FSP1 by FSP1 Inhibitor (iFSP1). Data are plotted as the mean of triplicate measurements ± the standard deviation. The vehicle control (Veh.) represents 100% initial activity. The average IC_{50} value of FSP1 Inhibitor (iFSP1) is 267 ± 47 nM.

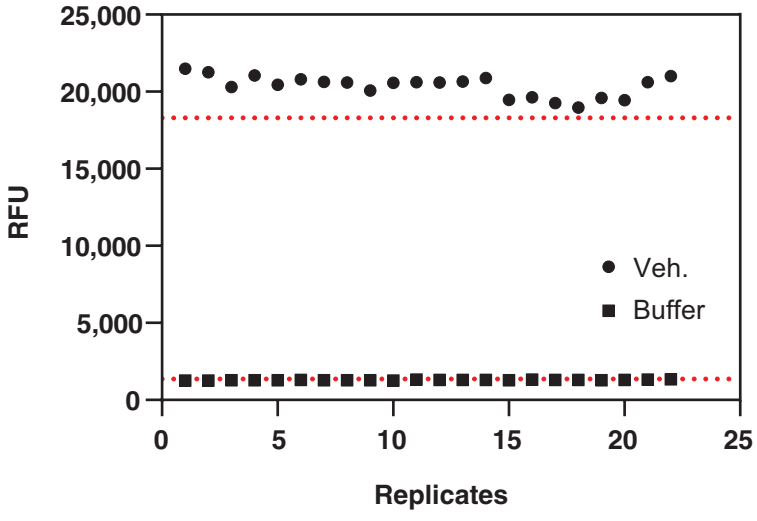

Figure 4. Typical Z′ data for the FSP1 Fluorescent Inhibitor Screening Assay Kit. Data are shown from 22 replicates each for vehicle control (Veh.) and buffer prepared as described in the kit booklet. The calculated Z′ factor for this experiment was 0.89. The red lines correspond to three standard deviations from the mean for each control value.

Effects of Solvents:

Compounds may be prepared in organic solvents such as DMSO, DMF, or short-chain alcohols (*e.g.*, MeOH, EtOH), as long as the final concentration of organic solvents in the assay is ≤5% for DMSO and ≤1% for DMF, MeOH, and EtOH. A titration of organic solvents showed that the signal decreases with increasing DMF, MeOH, and EtOH concentration so the proper vehicle control should be included in the assay.

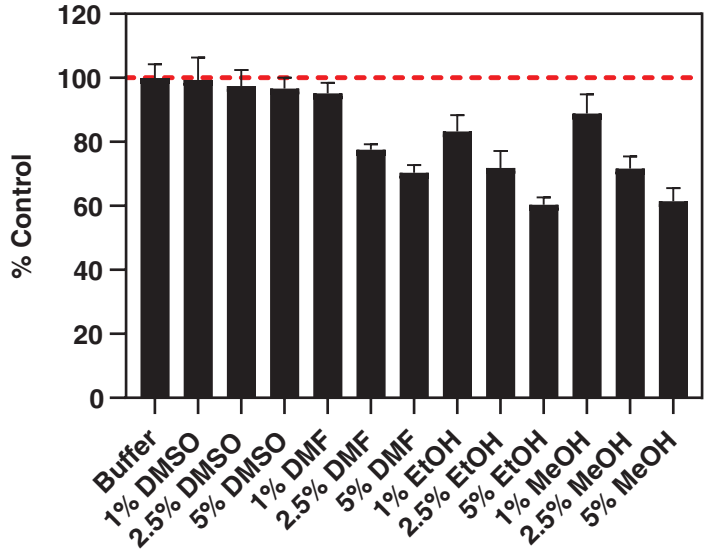

Figure 5. The effect of solvent on the readout of FSP1 activity. The data are shown as the mean ± standard error of the mean for triplicate reactions containing the indicated concentration of solvents.

Precision:

Intra-assay precision was determined by analyzing 8 measurements of the background, vehicle, and FSP1 Inhibitor (iFSP1) on the same day. The intra-assay coefficients of variation were 0.8, 5.5, and 5.6%, respectively. The intra-assay coefficient of variation for the IC_{50} value of 8 inhibition curves performed on the same day was 18.5%.

Inter-assay precision was determined by analyzing inhibition with FSP1 Inhibitor (iFSP1) in separate assays on four different days. The inter-assay coefficient of variance for the IC_{50} value was 12.1%.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	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 fluorescence detected above background in the inhibitor wells	A. Enzyme or substrate was not added to the well(s) B. Inhibitor concentration is too high and inhibited all of the enzyme activity	A. Make sure to add all the components to the well(s) B. Reduce the inhibitor concentration and re-assay
The fluorometer exhibited 'MAX' values for the wells	The *gain* setting is too high	Reduce the *gain* and re-read
No inhibition seen with compound	A. The compound concentration is not high enough B. The compound is not an inhibitor of the enzyme	Increase the compound concentration and re-assay

References

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2. Dixon, S.J. and Stockwell, B. The hallmarks of ferroptosis. *Annu. Rev. Anal. Chem.* **3(1)**, 35-54 (2019).
3. Cao, J.Y. and Dixon, S.J. Mechanisms of ferroptosis. *Cell. Mol. Life Sci.* **73(11-12)**, 2195-2209 (2016).
4. Doll, S., Freitas, F.P., Shah, R., *et al.* FSP1 is a glutathione-independent ferroptosis suppressor. *Nature* **575(7784)**, 693-698 (2019).
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6. Marshall, K.R., Gong, M., Wodke, L., *et al.* The human apoptosis-inducing protein AMID is an oxidoreductase with a modified flavin cofactor and DNA binding activity. *J. Biol. Chem.* **280(35)**, 30735-30740 (2005).
7. Zhang, J.-H., Chung, T.D.Y., and Oldenburg, K.R. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen.* **4(2)**, 67-73 (1999).

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.