Thromboxane A Synthase Inhibitor Screening Assay Kit

Item No. 702160

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size	Storage
400486	TXAS FP Assay Buffer	1 vial/20 ml	4°C
400487	TXAS Protein (human, recombinant)	2 vials	-20°C
400488	TXAS FP Probe	1 vial/150 µl	-20°C
400489	TXAS Inhibitor Positive Control	1 vial/50 µl	-20°C
700416	DTT (1 M) Assay Reagent	1 vial/1 ml	-20°C
10005371	384-Well Solid Plate (black; non-binding)	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

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 polarization (FP) with excitation and emission wavelengths of 530 and 590 nm, respectively
2. Adjustable pipettes; multichannel or repeating pipettor recommended
3. A source of pure water; glass-distilled water or deionized water is acceptable
   NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000)
4. Microcentrifuge tubes
5. Appropriate solvent for test compounds

INTRODUCTION

Background

Thromboxane A synthase (TXAS), also known as cytochrome P450 (CYP) isoform CYP5A1, is an enzyme that catalyzes the isomerization of prostaglandin H_2 (PGH_2) into thromboxane A_2 (TXA_2), a potent vasoconstrictor and inducer of platelet aggregation. TXA_2 is rapidly hydrolyzed non-enzymatically to the inactive metabolite TXB_2.[1,2] TXAS also catalyzes the cleavage of PGH_2 into malondialdehyde (MDA) and 12(S)-hydroxyheptadecatrienoic acid (12(S)-HHTrE), a leukotriene B_4 (LTB_4) receptor 2 (BLT_2) agonist.[1] Upon reaction with PGH_2, TXAS undergoes irreversible catalytic inactivation.[3] TXAS is expressed in platelets, monocytes, and macrophages, as well as several tissues, including the lungs, kidneys, stomach, and colon, and is localized to the endoplasmic reticulum.[3] Urinary levels of the TXB_2 metabolites 11-dehydro TXB_2 and 2,3-dinor TXB_2 are increased in patients with asthma, and elevated urinary levels of 11-dehydro TXB_2 are associated with an increased risk of a major adverse cardiovascular event in patients with atherosclerotic cardiovascular disease.[4-6]

About This Assay

Cayman's TXAS Inhibitor Screening Assay Kit provides a robust and easy-to-use platform for identifying novel inhibitors of human TXAS. The assay uses a TAMRA-conjugated TXAS-specific probe suitable for fluorescence polarization (FP)-based screening of TXAS inhibitors. Inhibitors of TXAS displace the fluorescent probe from the TXAS active site, leading to a decrease in FP that can be easily quantified using an FP-capable plate reader at excitation and emission wavelengths of 530 and 590 nm, respectively. The potent and reversible TXAS inhibitor ozagrel, or OKY-046, is included as a positive control and inhibits TXAS with an IC_{50} value of 25-75 nM.

NOTE: The binding affinity of an inhibitor is determined relative to a fluorescently labeled inhibitor. Therefore, the inhibition value determined in this assay may not give the same value as that determined in an enzymatic or cell-based assay. Nevertheless, a high correlation between assay techniques is typically observed.

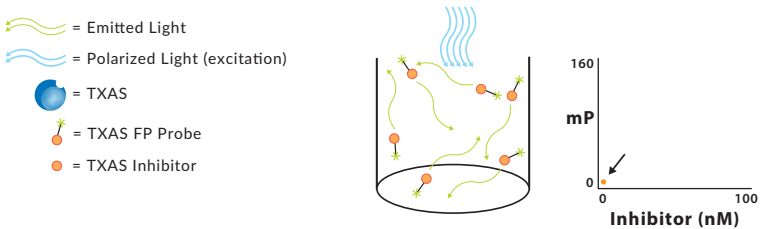
Introduction to Fluorescence Polarization

Fluorescence polarization (FP) assays are homogeneous, single-step assays ideally suited for high-throughput screening (HTS) of large numbers of samples. All FP assays employ a large molecular species, or binding partner, in conjunction with a small, low molecular weight fluorescent analyte (FA).

Fluorescence is, by definition, the ability of a molecule to absorb the energy of an incoming (excitation) photon and then re-emit most of this energy as a new, slightly less energetic (emission) photon.

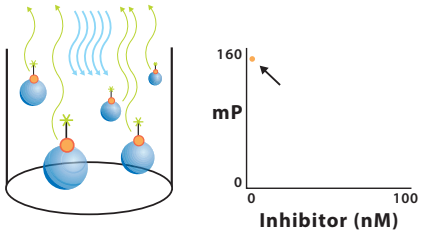
A small fluorescent molecule will rotate appreciably during the very small interval of time between absorption of a photon and emission of the fluorescence photon.

If the excitation light is polarized, this rotation will result in complete randomization of the plane of the emitted light. Thus, small fluorescent molecules depolarize an excitation pulse of polarized light.

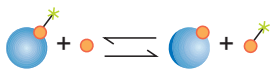


Large fluorescent molecules do not rotate appreciably in the same small interval of time. They will, therefore, emit light that retains some of the polarization of the polarized excitation light. This polarization is quantified as milli-polarization units, or mP. An FP reader is required to make this measurement.

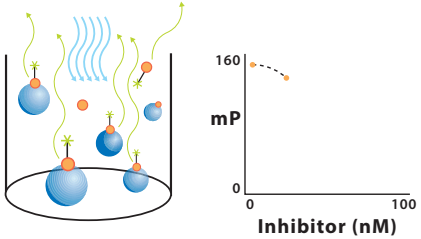
When a small fluorescent molecule becomes tightly bound to a large one, as in the binding of TXAS to the fluorescent probe, the rotational speed of the small molecule is abruptly reduced to that of the entire complex as a whole.



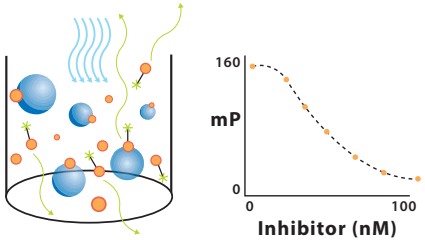
Therefore, the fluorescent probe bound to TXAS represents a large fluorescent molecule, which exhibits a high degree of FP. A microplate well filled with the fluorescent probe-TXAS complex will give a high FP reading. The TXAS Inhibitor Screening Assay Kit is based on the competition of free inhibitor in the samples or standards for the high-affinity binding site of TXAS occupied by the fluorescent probe. Addition of a small amount of TXAS inhibitor will result in the displacement of the fluorescent probe from the TXAS binding site.



Some of the fluorescent probe will be released from the TXAS and will resume its intrinsic, rapid rate of rotation. This will cause a detectable loss of FP in the well.



The addition of large amounts of a TXAS inhibitor will result in a much larger reduction in the mP of the well. Plotting mP *versus* inhibitor concentration allows the construction of an inhibition curve with a broad dynamic range.



Cayman's TXAS Inhibitor Screening Assay Kit allows for the rapid identification of TXAS inhibitors with a wide range of IC_{50} values.

PRE-ASSAY PREPARATION

Sample Preparation

All inhibitors, be they small molecules, natural products, or proteins, should be prepared in DMSO, dimethyl formamide (DMF), short-chain alcohols (*e.g.*, MeOH, EtOH), or an appropriate buffer at a concentration 20X the desired final assay concentration (*e.g.*, for 1 µM final assay concentration, a 20 µM stock should be made). The final concentration of inhibitor diluent in the assay will then be 5% (see **Effects of Solvents** on page 20).

Reagent Preparation

1. **TXAS FP Assay Buffer - (Item No. 400486)**

   Mix 20 ml of TXAS FP Assay Buffer with 0.1 ml of the supplied DTT (1 M) Assay Reagent (Item No. 700416) to make 20.1 ml of TXAS FP Assay Buffer + DTT. The resulting buffer will have a DTT concentration of 5 mM. After DTT has been added, the TXAS FP Assay Buffer + DTT should be used within the same day and kept on ice, otherwise, aliquot and store at -20°C where it will be stable for at least three months.

2. **TXAS Protein (human, recombinant) - (Item No. 400487)**

   Each vial contains a lyophilized powder of TXAS Protein (human, recombinant). Reconstitute the contents of the vial with 0.5 ml of pure water, mix gently, and place on ice. One vial provides a sufficient volume to assay 200 wells. The reconstituted TXAS Protein (human, recombinant) will be stable for four hours when stored on ice. If all of the reconstituted protein will not be used at one time, aliquot and store at -80°C. Avoid multiple freeze-thaw cycles.

3. **TXAS FP Probe - (Item No. 400488)**

   This vial contains 150 µl of TXAS FP Probe. The reagent is ready to use as supplied. If all of the TXAS FP Probe will not be used at one time, aliquot and store at -20°C where it will be stable for at least six months.

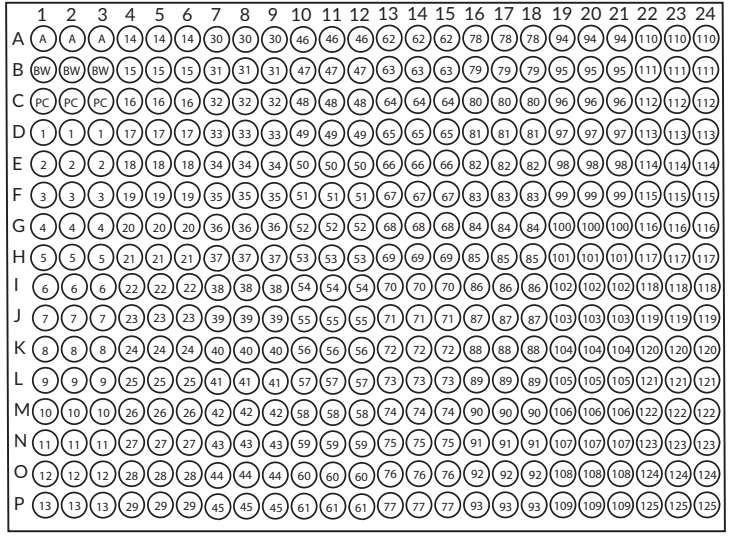
4. **TXAS Inhibitor Positive Control - (Item No. 400489)**

   This vial contains 50 µl of 2 mM TXAS Inhibitor Positive Control in DMSO. Mix 2 µl of TXAS Inhibitor Positive Control with 18 µl of DMSO to make a 200 µM working solution. If all of the TXAS Inhibitor Positive Control will not be used at one time, aliquot the undiluted inhibitor and store at -20°C, where it will be stable for at least six months. The diluted TXAS Inhibitor Positive Control may be stored at -20°C for up to three months.

ASSAY PROTOCOL

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 test compound, including the TXAS Inhibitor Positive Control, be assayed in triplicate. A typical layout of samples to be measured in triplicate is shown in Figure 1, below.



A = 100% Initial Activity Wells
BW = Background Wells
PC = Positive Control Wells
1-125 = Test Compound Wells

Figure 1. 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 the wells.
- All reagents should be prepared as described above. The reconstituted TXAS Protein (human, recombinant) and TXAS FP Assay Buffer + DTT 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.
- It is recommended to assay the samples in triplicate, but it is at the user's discretion to do so.
- The assay is performed at room temperature.
- Monitor the FP using an FP-capable plate reader with an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

Performing the Assay

1. Prepare sufficient volumes of TXAS+ and TXAS- Master Mixes according to the table below. Scale the volumes up or down as needed. Each well requires 47.5 µl of the corresponding master mix.

	TXAS+ Master Mix (For 100% Initial Activity, Test Compound, and Positive Control Wells)	TXAS- Master Mix (For Background Wells)
TXAS FP Assay Buffer + DTT	4,525 µl	189 µl
TXAS FP Probe	25 µl	1 µl
Reconstituted TXAS Protein (human, recombinant)	200 µl	--
Final Volume (# of wells)	4,750 µl (100 wells)	190 µl (4 wells)

Table 1. Master mix preparation

2. **Background Wells**: add 47.5 µl of the TXAS- Master Mix and 2.5 µl of DMSO (positive control diluent) to three wells. If test compounds in different diluents are to be assayed at the same time, separate sets of background wells should be added for each diluent used.

3. **100% Initial Activity Wells**: add 47.5 µl of the TXAS+ Master Mix and 2.5 µl of DMSO (positive control diluent) to three 100% initial activity wells. If test compounds in different diluents are to be assayed at the same time, separate sets of 100% initial activity wells should be added for each diluent used.
4. **Test Compound/Positive Control Wells**: add 47.5 µl of the TXAS+ Master Mix and 2.5 µl of test compound or the 200 µM TXAS Inhibitor Positive Control working solution to three wells.
5. Mix the contents of all wells by gently pipetting up and down at least four times. Avoid forming bubbles.
6. Cover the plate with the Foil Plate Cover (Item No. 400023) and incubate for one hour at room temperature.
7. Remove the plate cover and determine FP using an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

ANALYSIS

Calculations

1. Determine the average fluorescence polarization (AFP) of each sample.
2. Subtract the AFP of the background wells from the AFP of the 100% initial activity and inhibitor wells. These are the corrected values.
3. Graph the corrected values 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 TXAS by the TXAS Inhibitor Positive Control is shown in Figure 2 (see page 18).

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 TXAS Inhibitor Screening Assay Kit was determined to be 0.83 (see Figure 3, page 19).

Sample Data:

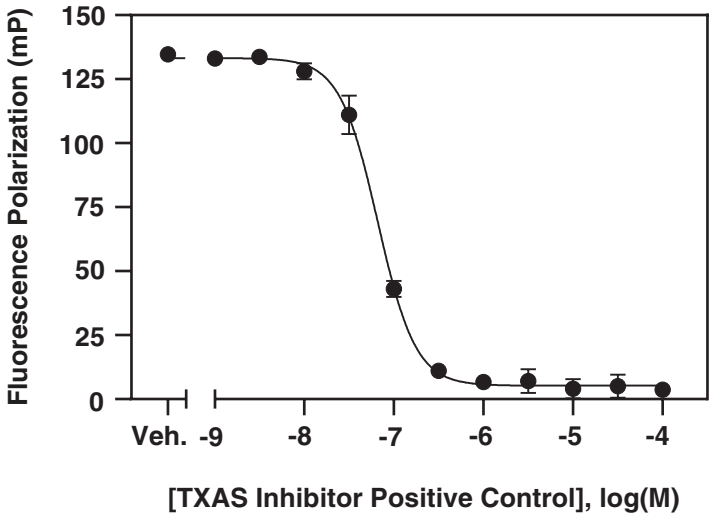


Figure 2. Inhibition of recombinant human TXAS by TXAS Inhibitor Positive Control. Data are plotted as the mean of triplicate measurements ± the standard deviation. The vehicle control (Veh.) represents 100% initial activity. The IC_{50} value of TXAS Inhibitor Positive Control in this example is 66 nM.

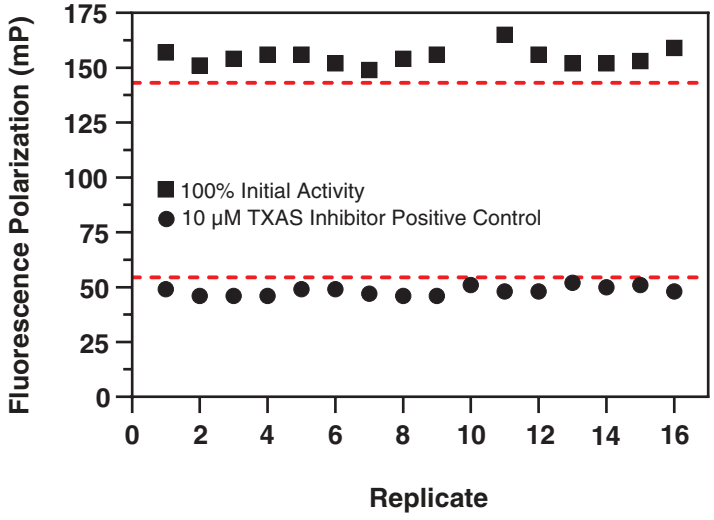


Figure 3. Typical performance data for the TXAS Inhibitor Screening Assay Kit. Data are shown from 15 replicates of 100% Initial Activity wells and 16 replicates of 10 µM TXAS Inhibitor Positive Control prepared as described in the kit booklet. The calculated Z′ factor for this experiment was 0.83. The 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, short-chain alcohols (*e.g.*, MeOH, EtOH), or an appropriate buffer. The final concentration of the diluent in the assay is 5%. A titration of organic solvents showed that signal can change with increasing solvent concentration so the proper vehicle control should be included in the assay.

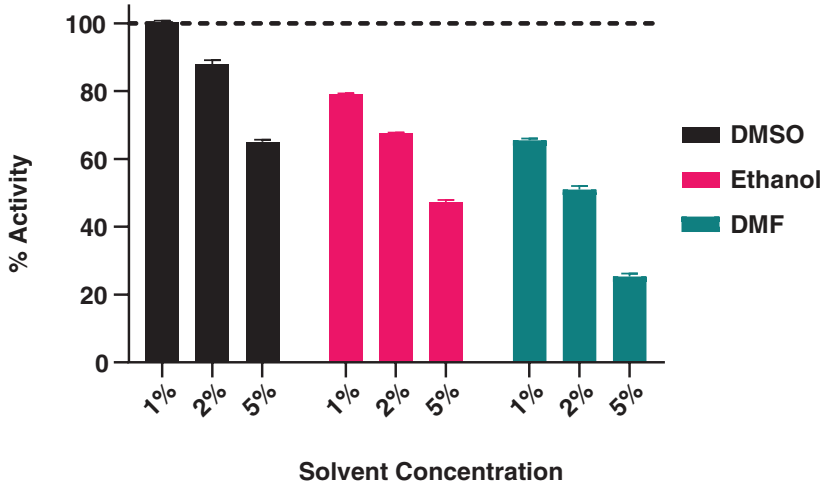


Figure 4. The effect of solvent on the readout of TXAS activity. The data are shown as the mean ± standard deviation for quadruplicate reactions containing the indicated concentration of solvents. The dotted line represents 100% activity (no solvent added).

Precision:

Intra-assay precision was determined by analyzing 15 measurements of the background, vehicle, and 10 µM TXAS Inhibitor Positive Control on the same day. The intra-assay coefficients of variation were 3, 4, and 7%, respectively. The intra-assay coefficient of variation for the IC_{50} value of 15 inhibition curves performed on the same day was 8%.

Inter-assay precision was determined by analyzing inhibition with TXAS Inhibitor Positive Control in six separate assays on three different days. The inter-assay coefficient of variance for the IC_{50} value was 9%.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
A. Erratic values B. Dispersion of duplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells or try more replicates of inhibitor standard to achieve consistency B. Carefully tap the side of the plate with your finger to remove the bubbles
High background mP	Dilution error	Check the dilution of each component

Reagent	Procedure
TXAS FP Assay Buffer + DTT	Add 0.1 ml of DTT (1M) Assay Reagent for every 20 ml of TXAS FP Assay Buffer to make TXAS FP Assay Buffer + DTT
TXAS Protein (human, recombinant)	Reconstitute contents of each vial in 0.5 ml of pure water
TXAS FP Probe	Ready to use as supplied
TXAS Inhibitor Positive Control	Dilute 1:10 with DMSO to make a 200 µM inhibitor working solution

Table 2. Assay reagent preparation summary

	TXAS+ Master Mix (For 100% Initial Activity, Test Compound, and Positive Control Wells)	TXAS- Master Mix (For Background Wells)
TXAS FP Assay Buffer + DTT	4,525 µl	189 µl
TXAS FP Probe	25 µl	1 µl
Reconstituted TXAS Protein (human, recombinant)	200 µl	--
Final Volume (# of wells)	4,750 µl (100 wells)	190 µl (4 wells)

Table 3. Master mix summary

Procedure	Background Wells	100% Activity Wells	Positive Control Wells	Test Compound Wells
Add TXAS+ Master Mix	--	47.5 µl	47.5 µl	47.5 µl
Add TXAS- Master Mix	47.5 µl	--	--	--
Add diluent	2.5 µl	2.5 µl	--	--
Add 200 µM TXAS Inhibitor Positive Control	--	--	2.5 µl	--
Add Test Compound (20X)	--	--	--	2.5 µl
Mix and Incubate	Mix by gentle pipetting. Cover and incubate at room temperature for 60 minutes protected from light.			
Read	Read fluorescence polarization at excitation and emission wavelengths of 530 nm and 590 nm, respectively.			

Table 4. 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.