COX Fluorescent Activity Assay Kit

Item No. 700200

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Kit components may be stored at -80°C prior to use. After opening the kit, we recommend each kit component be stored according to the temperature listed below.

Item Number	Item	Quantity	Storage
760114	Assay Buffer (10X)	2 vials	-20°C
760116	Hemin	1 vial	-20°C
700110	COX Positive Control	1 vial	-80°C
760113	Arachidonic Acid (substrate)	1 vial	-80°C
760115	Potassium Hydroxide	1 vial	-20°C
700002	ADHP Assay Reagent	4 vials	-20°C
760158	DuP-697	1 vial	-20°C
760159	SC-560	1 vial	-20°C
700001	DMSO Assay Reagent	1 vial	RT
700023	Resorufin Standard	100 µl	-20°C
400017	96-Well Solid Plate (black)	2 plates	RT
400012	Plate Cover	2 covers	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.

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, on 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 using an excitation wavelength between 530-540 nm and an emission wavelength between 585-595 nm
2. Adjustable pipettes and a multichannel or repeating pipette
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

Cyclooxygenase (COX, also called Prostaglandin H Synthase or PGHS) is a bifunctional enzyme exhibiting both COX and peroxidase activities. The COX component converts arachidonic acid to a hydroperoxy endoperoxide (PGG_2), and the peroxidase component reduces the endoperoxide to the corresponding alcohol (PGH_2), the precursor of PGs, thromboxanes, and prostacyclins.[1,2]

It is now well established that there are two distinct isoforms of COX. COX-1 is constitutively expressed in a variety of cell types and is involved in normal cellular homeostasis. A variety of mitogenic stimuli such as phorbol esters, lipopolysaccharides, and cytokines lead to the induced expression of a second isoform of COX, COX-2. COX-2 is responsible for the biosynthesis of PGs under acute inflammatory conditions.[3,4] This inducible COX-2 is believed to be the target enzyme for the anti-inflammatory activity of nonsteroidal anti-inflammatory drugs.[4]

About This Assay

Cayman's COX Fluorescent Activity Assay provides a convenient fluorescence-based method for detecting COX-1 or COX-2 activity in both crude (cell lysates/tissue homogenates) and purified enzyme preparations. The assay utilizes the peroxidase component of COXs. The reaction between PGG_2 and ADHP (10-acetyl-3,7-dihydroxyphenoxazine) produces the highly fluorescent compound resorufin. Resorufin fluorescence can be easily analyzed with an excitation wavelength between 530-540 nm and an emission wavelength between 585-595 nm (see Figure 1 on page 7). The kit includes isozyme-specific inhibitors for distinguishing COX-2 activity from COX-1 activity.

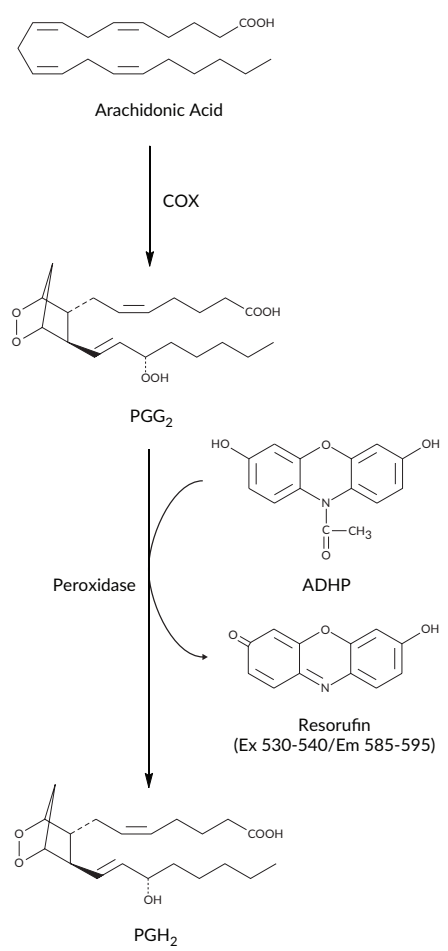


Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Assay Buffer (10X) - (Item No. 760114)**

   Mix 3 ml of Assay Buffer concentrate with 27 ml of HPLC-grade water. This final Buffer (100 mM Tris-HCl, pH 8.0) should be used in the assay and for diluting reagents. When stored at 4°C, this 1X Assay Buffer is stable for at least six months.

2. **Hemin - (Item No. 760116)**

   This vial contains 300 µl of Hemin in DMSO. Mix 40 µl of Hemin with 960 µl of 1X Assay Buffer. The diluted Hemin is stable for 12 hours at room temperature.

3. **COX Positive Control - (Item No. 700110)**

   This vial contains ovine COX-1 and should be kept on ice when thawed. Mix 10 µl of enzyme with 490 µl of 1X Assay Buffer and store on ice. The diluted enzyme is stable for one hour.

4. **Arachidonic Acid - (Item No. 760113)**

   This vial contains 400 µl of Arachidonic Acid in ethanol. Transfer 100 µl of the supplied solution to another vial, add 100 µl of KOH (Item No. 760115), vortex, and then add 800 µl of HPLC-grade water to achieve a final stock of 2 mM substrate. Use the prepared Arachidonic Acid solution within 30 minutes. A 10 µl aliquot will yield a final concentration of 100 µM in the wells. If a lower concentration is desired, dilute further with HPLC-grade water and use within 30 minutes.

5. **Potassium Hydroxide - (Item No. 760115)**

   This vial contains 500 µl of Potassium Hydroxide (KOH). The reagent is ready to use as supplied.

6. **ADHP Assay Reagent - (Item No. 700002)**

   These vials contain a clear lyophilized powder of 10-acetyl-3,7-dihydroxyphenoxazine (ADHP). Immediately prior to assaying, dissolve the contents of one vial in 100 µl of DMSO Assay Reagent (Item No. 700001), and then add 400 µl of 1X Assay Buffer. This is enough substrate to assay 50 wells. Prepare additional vials as needed. The reconstituted substrate is stable for 30 minutes. After 30 minutes, increased background fluorescence will occur.

7. **DuP-697 - (Item No. 760158)**

   This vial contains 60 µM of DuP-697. DuP-697 is a potent and time-dependent inhibitor of COX-2.[5] The reagent is ready to use as supplied.

8. **SC-560 - (Item No. 760159)**

   This vial contains 66 µM of SC-560. SC-560 is a potent and selective COX-1 inhibitor.[6] The reagent is ready to use as supplied.

9. **DMSO Assay Reagent - (Item No. 700001)**

   This vial contains 1 ml of Dimethylsulfoxide (DMSO). The reagent is ready to use as supplied.

10. **Resorufin Standard - (Item No. 700023)**

    This vial contains 100 µl of Resorufin in DMSO. It is ready to use to prepare the standard curve.

Sample Preparation

Tissue Homogenate

1. Prior to dissection, rinse tissue with PBS (phosphate buffered saline solution, pH 7.4) to remove any red blood cells and clots.
2. Homogenize the tissue in 5-10 ml of cold buffer (*i.e.*, 100 mM Tris-HCl, pH 7.5, containing protease inhibitors of choice; see **Interferences** on page 23) per gram weight of tissue.
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

Cell Lysate

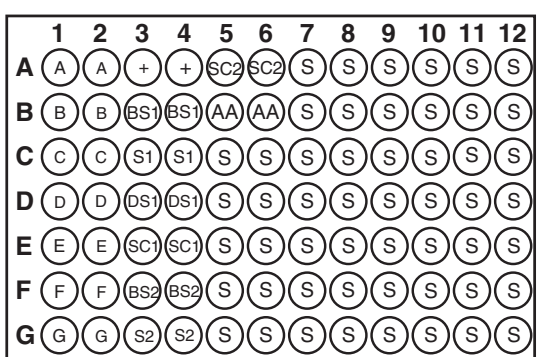
1. Collect cells (~5 x 10^6) by centrifugation (*i.e.*, 1,000-2,000 x g for 10 minutes at 4°C). For adherent cells, do not harvest using proteolytic enzymes; rather use a rubber policeman.
2. Sonicate cell pellet in 0.5-1 ml of cold buffer (*i.e.*, 100 mM Tris-HCl, pH 7.5, containing protease inhibitors of choice; see **Interferences** on page 23).
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

NOTE: The assay does not work with plasma/serum samples.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. However, a resorufin standard curve in duplicate must be assayed with the sample along with two wells for the COX positive control. We suggest that each sample be assayed at least in duplicate and to have two wells designated as background wells for each sample. To account for the flurorescence of the arachidonic acid in the presence of hemin and ADHP, we recommend adding two wells for the substrate background. We also recommend assaying each sample in the presence and absence of at least one inhibitor to determine which COX enzyme is present in the sample. Record the contents of each well on the template sheet provided on page 26. A typical layout of samples to be measured in duplicate is shown on page 12 (see Figure 2).



A-H = Resorufin Standards
+ = COX Positive Control
S = Other Samples
BS1 & BS2 = Background Samples 1 & 2
S1 & S2 = Samples 1 & 2
DS1 & DS2 = DuP-697-treated Samples 1 & 2
SC1 & SC2 = SC-560-treated Samples 1 & 2
AA = Substrate Background

Figure 2. Sample plate format

Standard Preparation

Mix 20 µl of the Resorufin Standard (Item No. 700023) with 3.98 ml of 1X Assay Buffer to yield a 10 µM Resorufin stock. Label eight tubes A-H. Add the amount of Resorufin stock (10 µM) and 1X Assay Buffer to each tube as described in Table 1. The diluted Standards are stable for four hours at room temperature.

Tube	10 µM Resorufin stock (µl)	1X Assay Buffer (µl)	Final concentration (µM)
A	0	1,000	0
B	50	950	0.5
C	100	900	1
D	200	800	2
E	400	600	4
F	600	400	6
G	800	200	8
H	1,000	0	10

Table 1. Preparation of Resorufin Standards

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells.
- 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 190 µl in all the wells.
- Use the 1X Assay Buffer in the assay.
- All reagents except the COX Positive Control and samples must be equilibrated to room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- You do not have to use both inhibitors (DuP-697 and SC-560). It is the user's discretion to include these inhibitors in the assay.
- It is recommended to assay 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 between 530-540 nm and an emission wavelength between 585-595 nm.

Performing the Assay

Read all of these instructions before setting up your plate!

NOTE: Initiate the reactions with arachidonic acid while near the plate reader as the plate must be read after a one minute incubation period.

1. **Standard Wells** - add 180 µl of 1X Assay Buffer and 10 µl of Standard (tubes A-H) to the designated wells on the plate (see **Sample Plate Format**, Figure 2, page 12).
2. Read the plate after five minutes using an excitation wavelength between 530-540 nm and an emission wavelength between 585-595 nm. Reading the standards prior to measuring sample activity allows an appropriate *gain* to be established for detecting the entire range of the standards. This *gain* must also be used when assaying the samples.
3. **COX Positive Control Wells** - add 150 µl of 1X Assay Buffer, 10 µl of Hemin, and 10 µl of COX to two wells.
4. **Sample Wells** - add 150 µl of 1X Assay Buffer, 10 µl of Hemin, and 10 µl of sample to two wells. To obtain reproducible results, the amount of COX added to the wells should fall within the range of the assay. When necessary, samples should be diluted with 1X Assay Buffer or concentrated using a spin concentrator with a molecular weight cut-off of 30 kDa to bring the enzymatic activity to this level.
5. **Sample Background Wells** - add 160 µl of 1X Assay Buffer, 10 µl of Hemin, and 10 µl of sample to two wells.
6. **Substrate Background Wells** - add 160 µl of 1X Assay Buffer and 10 µl of Hemin to two wells.
7. **Inhibitor Wells** - add 140 µl of 1X Assay Buffer, 10 µl of Hemin, and 10 µl of sample to two wells. Add either 10 µl of DuP-697 or SC-560 to the two wells (see **Sample Plate Format**, Figure 2, page 12). *NOTE: DuP-697 will diminish COX-2 activity and SC-560 will diminish COX-1 activity. If you already know which COX isoform (COX-1 or COX-2) is present in your sample, you can skip this step. If it is unknown which isoform is present, then you can pick either inhibitor or use an additional two wells and assay activity with both inhibitors.*

8. If including inhibitors, incubate the plate for five minutes at room temperature
9. Add 10 µl of ADHP to each reaction.
10. Initiate the reactions by **quickly** adding 10 µl of Arachidonic Acid Solution to the Positive Control, sample, substrate background, and inhibitor wells only. DO NOT add Arachidonic Acid to the sample background wells.
11. Read the plate after **one minute** using an excitation wavelength between 530-540 nm and an emission wavelength between 585-595 nm.

Wells	Reagent (µl)							
	1X Assay buffer	Resorufin Standards	Hemin	Positive Control	Sample	Inhibitor	ADHP	Substrate (Arachidonic Acid)
Standard	180	10						
COX Positve Control	150		10	10			10	10
Sample	150		10		10		10	10
Sample Background	160		10		10		10	
Inhibitor treated samples	140		10		10	10	10	10
Substrate Background	160		10				10	10

Table 2. Assay set-up

ANALYSIS

Calculations

1. Determine the average fluorescence of each sample.
2. Subtract the average fluorescence of the sample background wells or the substrate background wells, whichever is higher, from the average fluorescence of the sample and the corresponding inhibitor wells to yield the corrected sample fluorescence (CSF).
3. Determine the average fluorescence of the standards. Subtract the fluorescence value of standard A (0 µM) from itself and all other standards. This is the corrected fluorescence.
4. Plot the corrected fluorescence values (from step 3 above) of each standard as a function of the final concentration of resorufin from Table 1. See Figure 3, on page 19, for a typical standard curve.
5. Calculate the fluorophore concentration of the samples using the equation obtained from the linear regression of the standard curve substituting the corrected fluorescence for each sample.

$$\text{Fluorophore concentration (µM)} = \frac{[\text{CSF} - (\text{y-intercept})]}{\text{Slope}}$$

6. Calculate the Total COX Activity using the following equation. One unit is defined as the amount of enzyme that will cause the formation of 1 nmol of fluorophore per minute at 22°C.

$$\text{Total COX Activity (nmol/min/ml)} = \left[\frac{\mu M}{\text{Minute}} \right] \times \text{Sample dilution}$$

7. Subtract the Total COX Activity of each Inhibitor-treated Sample from the Total COX Activity of its corresponding Sample. Divide by the Total COX Activity of the Sample and multiply by 100 to give the percent inhibition. The amount of inhibition corresponds to the amount of either COX-1 or COX-2 in the sample (see Table 2, page 20, for some examples).

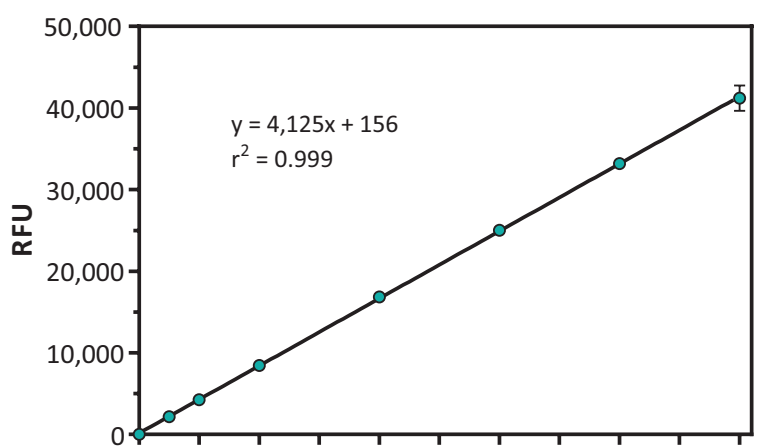


Figure 3. Resorufin standard curve
NOTE: The actual slope will vary depending on gain settings and equipment variances.

Sample	Total COX Activity	COX-1 (%)	COX-2 (%)
Sample 1	10	Unknown	Unknown
DuP-697 treated	0	0	100
SC-560 treated	10	0	100
Sample 2	20	Unknown	Unknown
DuP-697 treated	20	100	0
SC-560 treated	0	100	0
Sample 3	30	Unknown	Unknown
DuP-697 treated	15	50	50
SC-560 treated	15	50	50
Sample 4	20	Unknown	Unknown
DuP-697 treated	5	25	75
SC-560 treated	15	25	75

Table 3. Interpreting sample data

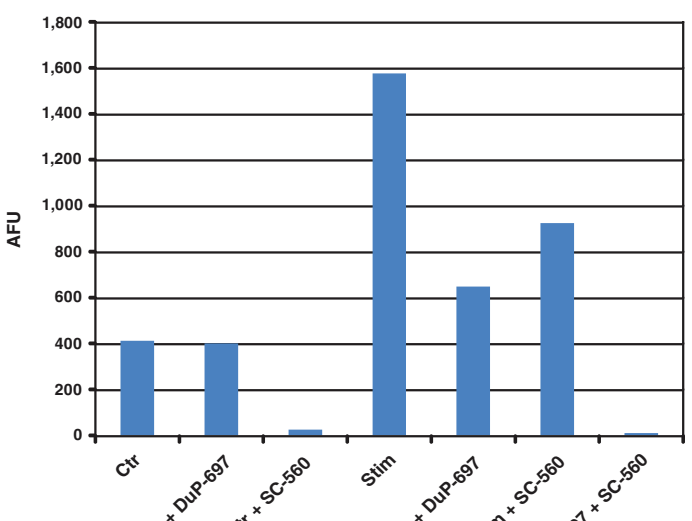


Figure 4. COX activity in murine macrophage cell lysates
COX activity was detected in LPS/IFNγ-stimulated RAW (murine macrophage) cell lysates. Cells (5 x 10^6) were induced with 200 ng LPS and 200 U/ml IFNγ for 24 hours. Cell pellets were suspended in 1 ml of 0.1 M Tris-HCl (pH 7.5) and lysed by sonication. The lysed cells were centrifuged at 10,000 x g at 4°C for 10 minutes and the supernatant was assayed. COX fluorescence was compared to cells that were not treated with LPS/IFNγ (Control = Ctr). Control cells contained COX-1 and stimulated cells contained both the constitutive COX-1 and the induced COX-2. (Ctr = Control; Stim = Stimulated with LPS/IFNγ).

Performance Characteristics

Precision:

When a series of ten COX measurements were performed on the same day, the intra-assay coefficient of variation was 2.2%. When a series of ten COX measurements were performed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 2.3%.

Sensitivity:

Samples containing COX activity between 0.023-2 nmol/min/ml can be assayed without further dilution or concentration.

Interferences

The following reagents were tested in the assay for interference in the assay:

Reagent		Will Interfere (Yes or No)
Buffers	Tris	No
	HEPES	No
	Phosphate	No
Detergents	SDS (0.1%)	No
	Polysorbate 20 (≤1%)	No
	Triton X-100 (0.1%)	No
	Triton X-100 (1%)	Yes
Protease Inhibitors/ Chelators/ Enzymes	EDTA (1 mM)	No
	EGTA (1 mM)	No
	Trypsin (10 µg/ml)	No
	PMSF (1 mM)	No
	Leupeptin (10 µg/ml)	No
	Antipain (10 µg/ml)	No
	Chymostatin (10 µg/ml)	No
Solvents	Ethanol (10 µl)	No
	Methanol (10 µl)	No
	Dimethylsulfoxide (10 µl)	No
Others	BSA (0.1%)	No
	Glutathione (1 mM)	Yes
	Glycerol (5%)	No

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 sample wells	Sample was too dilute	Re-assay with a more concentrated sample
The fluorometer exhibited 'MAX' values for the wells	The *gain* setting is too high	Reduce the *gain* and re-read
No inhibition seen with the inhibitors (DuP-697 or SC-560)	A. The COX activity is too low to detect B. The sample does not contain COX (COX-1 or COX-2), or the sample contains something that is interfering	A. Re-assay with a more concentrated sample B. Check the interference section for possible Interferences (see page 24)

References

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2. Hamberg, M. and Samuelsson, B. Detection and isolation of an endoperoxide intermediate in prostaglandin biosynthesis. *Proc. Natl. Acad. Sci. USA* **70**, 899-903 (1973).
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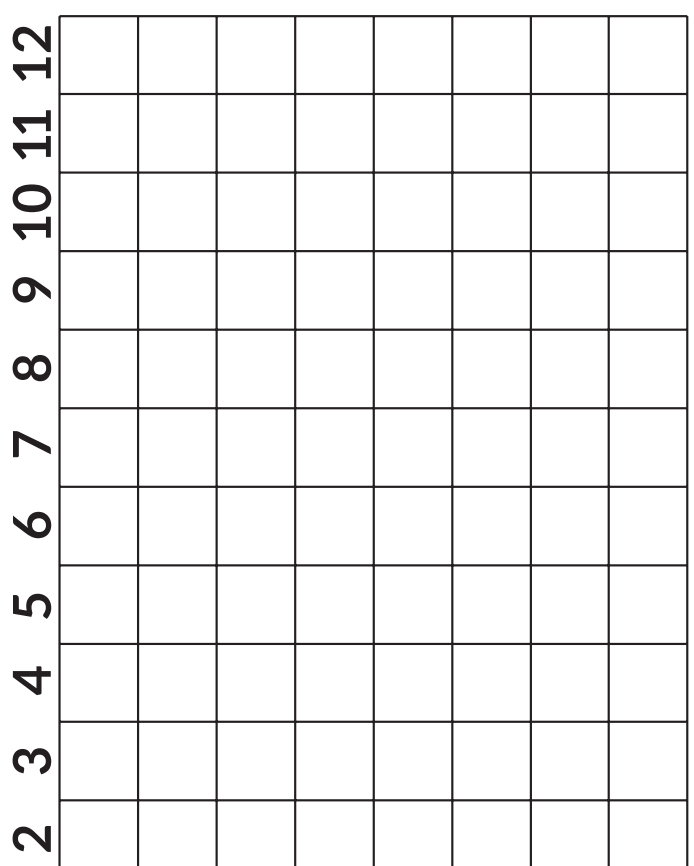
6. Smith, C.J., Zhang, Y., Koboldt, C.M., *et al.* Pharmacological analysis of cyclooxygenase-1 in inflammation. *Proc. Natl. Acad. Sci. USA* **95**, 13313-13318 (1998).



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.