NADPH/NADP Ratio Assay Kit

Item No. 702450

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size	Storage
400462	Extraction Buffer N	1 vial/50 ml	-20°C
400463	NADPH Reaction Buffer	1 vial/20 ml	-20°C
400464	NADPH Standard	1 vial	-20°C
400466	Developer W1	1 vial/1.5 ml	-20°C
400781	Developer W2	1 vial/150 µl	-20°C
400467	NADPH Converter Enzyme	1 vial	-20°C
400014	96-Well Solid Plate (Colorimetric Assay)	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.

The reagents in this kit have been tested and formulated to work exclusively with Cayman Chemical's NADPH/NADP Ratio Assay Kit. 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 absorbance at 450 nm
2. An orbital microplate shaker
3. Adjustable pipettes; multichannel or repeating pipettor recommended
4. A source of pure water; glass-distilled is acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
5. A heat block or water bath capable of heating to 60°C
6. Aluminum foil
7. Materials used for **Sample Preparation** (see page 10)

INTRODUCTION

Background

NADPH and $NADP^+$ are key cofactors in various biological enzymatic reactions with roles in diverse cellular functions, including cell survival, the maintenance of redox status, and intracellular signaling.[1-4] Cells maintain NADPH primarily in its reduced form to drive reactive oxygen species (ROS) detoxification and reductive biosynthesis, an anabolic pathway that reduces carbon atoms in metabolic intermediates during the synthesis of proteins, DNA, and lipids.[5,6] The ratio of NADPH to $NADP^+$ is subject to both spatial and temporal redistribution within cells and tissues to facilitate responses to feeding and fasting, exercise, circadian rhythm, diet, cell proliferation status, and disease states, including in diabetes and cancer.[5,7] It is also responsive to differing pH environments across organs and organelles to promote specific metabolic pathways. The NADPH/$NADP^+$ reduction potential is regulated primarily by the pentose phosphate, isocitrate dehydrogenase, malic enzyme, and one-carbon metabolism pathways in the cytosol and the malic enzyme, isocitrate dehydrogenase, transhydrogenase, and one-carbon metabolism pathways in the mitochondria.[5]

About This Assay

Cayman's NADPH/NADP Ratio Assay Kit provides a colorimetric method for measuring $NADP^+$, NADPH, and their ratio in cell culture extracts. In this assay, $NADP^+$ is enzymatically converted to NADPH by glucose-6-phosphate dehydrogenase (G6PDH), which leads to the reduction of the tetrazolium salt substrate WST-8 to a highly colored formazan that absorbs at 450 nm (see Figure 1, below). The amount of formazan produced is proportional to the amount of $NADP^+$ and NADPH in the matrix. To measure NADPH specifically, samples are heated to degrade $NADP^+$. The $NADP^+$ concentration is then calculated by subtracting the signal obtained from the heated sample from that of the corresponding non-heated sample. This assay has a range of 0-1.5 µM and a lower limit of detection (LLOD) of 0.02 µM.

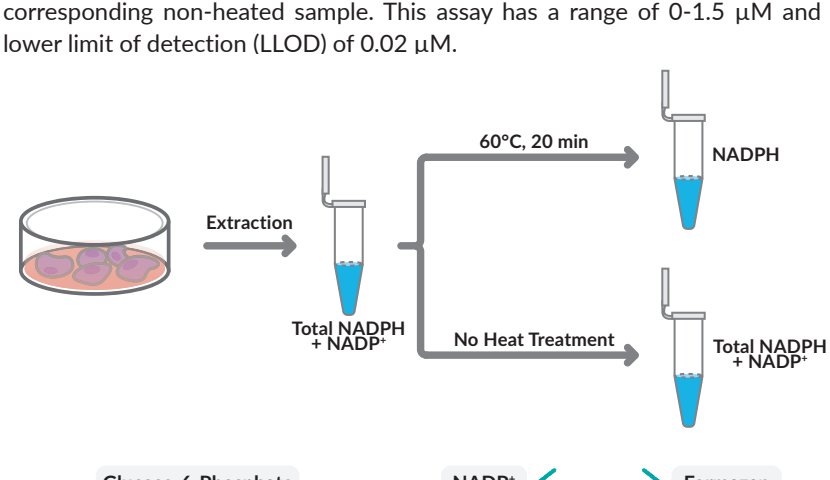

Figure 1. Sample extraction and assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Extraction Buffer N - (Item No. 400462)**

   This vial contains 50 ml of Extraction Buffer N. Thaw at room temperature and briefly vortex to ensure contents are in solution. Store thawed reagent on ice until use. If not using all at once, aliquot and store at -20°C.

2. **NADPH Reaction Buffer (1X) - (Item No. 400463)**

   This vial contains 20 ml of NADPH Reaction Buffer (1X). Thaw at room temperature and briefly vortex to ensure contents are in solution. Store thawed reagent on ice until use. The reaction buffer will be stable for 6 months when stored at -20°C.

3. **NADPH Standard - (Item No. 400464)**

   This vial contains lyophilized NADPH. Reconstitute the contents of the vial with 1 ml of pure water to obtain a solution of 200 µM NADPH. The reconstituted standard will be stable for four hours when stored on ice. If not using all at once, prepare aliquots and store at -20°C, where it will be stable for one month. Limit freeze-thaw cycles to one.

   Dilute 25 µl of the 200 µM NADPH Standard with 975 µl of Extraction Buffer N to yield a 5 µM bulk standard, which will be used to prepare the standards. The bulk standard will be stable for 4 hours at 4°C.

4. **NADPH Converter Enzyme - (Item No. 400467)**

   This vial contains lyophilized G6PDH. Reconstitute the contents of the vial with 1.5 ml of NADPH Reaction Buffer (1X) and place on ice. Do not vortex. The reconstituted enzyme will be stable for 4 hours when stored at 4°C. If not using the reconstituted enzyme all at once, prepare aliquots and store at -20°C, where it will be stable for 3 months. Limit freeze-thaw cycles to one.

5. **Developer W1 - (Item No. 400466)**

   This vial contains 1.5 ml of Developer W1. Thaw the vial at room temperature and vortex briefly to ensure contents are in solution. Store reagent on ice until use. If not using the Developer W1 all at once, prepare aliquots and store at -20°C. Limit freeze-thaw cycles to one.

6. **Developer W2 - (Item No. 400781)**

   This vial contains 150 µl of Developer W2. Thaw the vial at room temperature and vortex briefly to ensure contents are in solution. If not using the Developer W2 all at once, prepare aliquots and store at -20°C. Limit freeze-thaw cycles to one.

7. **NADPH Developer Reagent Preparation**

   To generate the NADPH Developer Reagent, combine the following reagents as shown in the table below and store on ice until use. NADPH Developer Reagent should be used within 1 hour of preparation. Scale accordingly. A surplus is prepared to account for volume transfer loss.

Component	50 Wells	100 Wells
NADPH Reaction Buffer	8,340 µl	16,680 µl
Reconstituted NADPH Converter Enzyme	600 µl	1,200 µl
Developer W1	600 µl	1,200 µl
Developer W2	60 µl	120 µl
Final Volume	9,600 µl	19,200 µl

Table 1. NADPH developer reagent preparation

Sample Preparation

Extract cells immediately after collection. $NADP^+$ and NADPH are highly labile and may degrade if extraction is delayed. Work quickly and keep samples on ice during and after extraction. If samples will not be assayed immediately, complete the extraction procedure and store at -80°C. To fall within the range of the standard curve, it may be necessary to dilute samples with Extraction Buffer N prior to performing the assay.

Suspension Cells

1. Pellet cells (~1 x 10^6 cells per sample replicate) by centrifugation (*i.e.* 1,000-2,000 x g for 10 minutes at 4°C).
2. Rapidly remove medium and extract cells with 200 µl of ice-cold Extraction Buffer N per 10^6 cells.
3. Transfer to a microcentrifuge tube and vortex for 10-20 seconds.
4. Keep on ice.

Adherent Cells

1. Grow cells to >80% confluency.
2. Place the culture dish/plate on ice, then rapidly remove the medium.
3. Quickly add ice-cold Extraction Buffer N. The recommended volumes of Extraction Buffer N are shown in the table below.

60 mm Dish	100 mm Dish	25 cm^2 Flask	75 cm^2 Flask
450 µl	1,000 µl	500 µl	1,500 µl

4. Collect the extract with a cell scraper.
5. Transfer the extract to a microcentrifuge tube and vortex for 10-20 seconds.
6. Keep on ice.

Sample preparation for exclusive measurement of NADPH

NOTE: This step is not necessary if only measuring the Total (NADPH and $NADP^+$). Transfer half the volume (minimum of 100 µl) of sample extract to a new microcentrifuge tube and heat to 60°C for 20 minutes in a heat block or water bath. $NADP^+$ is decomposed by heating, while NADPH remains intact. Place the heated sample on ice until performing the assay.

Sample Matrix Properties

Spike and Recovery

A549 cells were processed as described in the Sample Preparation section, spiked with equal amounts of NADPH and $NADP^+$, serially diluted with Extraction Buffer N, and evaluated using the NADPH/NADP Ratio Assay Kit. The results are shown in Figure 2 and Table 2. The error bars represent standard deviations obtained from multiple dilutions of each sample.

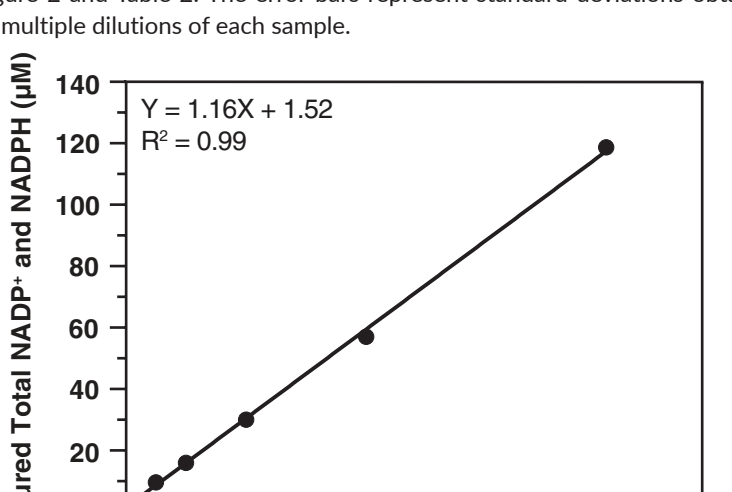

Figure 2. Spike and recovery of NADPH and $NADP^+$ in A549 cell extracts

Spike (µM)		Measured Total NADPH and $NADP^+$ (µM)	Measured NADPH (µM)	Calculated $NADP^+$ (µM)
NADPH	$NADP^+$			
50	50	118.7	58.1	60.6
25	25	57.1	28.1	29.0
12.5	12.5	30.0	15.0	15.0
6.25	6.25	16.0	8.8	7.2
3.125	3.125	9.7	5.6	4.1
0	0	2.3	1.6	0.7

Table 2. Spike and recovery table

Parallelism

To assess parallelism, A549 and HEK293T cell extracts were assayed at multiple dilutions using the NADPH/NADP Ratio Assay Kit. Samples were prepared as described in the **Sample Preparation** section (pages 10-11). Concentrations were plotted as a function of sample dilution.

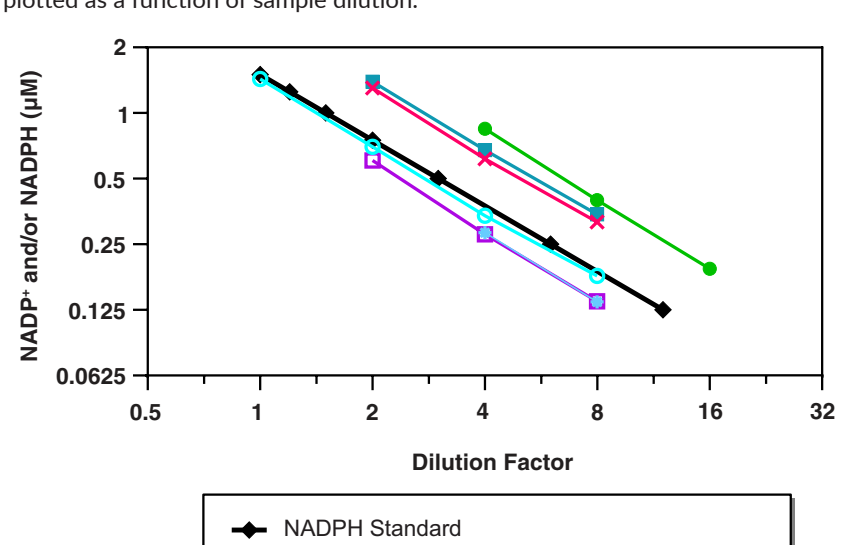

Figure 3. Parallelism of NADPH and NADP+ in A549 and HEK293T cell extracts

Linearity

A549 cells were prepared as described in the **Sample Preparation** section (pages 10-11) and then spiked with NADPH and NADP+, serially diluted with Extraction Buffer N, and evaluated for linearity using the NADPH/NADP Ratio Assay Kit.

Dilution	Measured Total NADPH + NADP+ (µM)	Linearity
Cell extract spiked with 50 µM NADPH and 50 µM NADP+		
100	120	100%
200	116	97%
400	118	99%
800	121	101%
Cell extract spiked with 25 µM NADPH and 25 µM NADP+		
50	59.3	100%
100	56.6	95%
200	55.3	93%

Table 3. Linearity of NADP+ and NADPH in A549 cell extract

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. It is suggested that each sample and standard be assayed at least in duplicate (triplicate is preferred). A typical layout of standards and samples to be measured in duplicate is shown in Figure 4. It is suggested that the contents of each well are recorded on the template sheet provided (see page 26).

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S1	S1	S9	S9	S17	S17	H5	H5	H13	H13
B	B	B	S2	S2	S10	S10	S18	S18	H6	H6	H14	H14
C	C	C	S3	S3	S11	S11	S19	S19	H7	H7	H15	H15
D	D	D	S4	S4	S12	S12	S20	S20	H8	H8	H16	H16
E	E	E	S5	S5	S13	S13	H1	H1	H9	H9	H17	H17
F	F	F	S6	S6	S14	S14	H2	H2	H10	H10	H18	H18
G	G	G	S7	S7	S15	S15	H3	H3	H11	H11	H19	H19
H	H	H	S8	S8	S16	S16	H4	H4	H12	H12	H20	H20

A-H = Standard Wells
S1-S20 = Sample Wells (Total)
H1-H20 = Heated Sample Wells (NADPH)

Figure 4. 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 in the assay is 200 µl in all of the wells.
- All reagents should be prepared as described above. Store the reconstituted reagents on ice until the assay.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended that the samples be assayed at least in duplicate, but it is at the user's discretion to do so.
- The assay is performed at room temperature.
- Measure the absorbance at 450 nm.

Standard Curve Preparation

NOTE: Prior to preparing the standards, ensure that the NADPH Standard has been diluted as instructed on page 8.

Label eight clean test tubes A-H. Add the amount of NADPH stock solution (5 µM) and Extraction Buffer N to each tube as described in Table 4. Mix thoroughly. The diluted standards will be stable for 4 hours at 4°C.

	Extraction Buffer N (µl)	5 µM NADPH Stock Solution (µl)	NADPH Standard (µM)
A	350	150	1.5
B	375	125	1.25
C	400	100	1
D	425	75	0.75
E	450	50	0.5
F	475	25	0.25
G	487.5	12.5	0.125
H	500	0	0

Table 4. Preparation of the NADPH standards

Performing the Assay

1. Add the reagents to the designated wells following the table below.
2. Cover the plate loosely with aluminum foil and incubate for 60 minutes at room temperature.
3. Remove the foil and read absorbance at 450 nm.

Reagent	Standard Wells	Heated Sample Wells (NADPH)	Sample Wells (Total)
Standards	40 µl	--	--
Heated Sample	--	40 µl	--
Non-heated Sample	--	--	40 µl
NADPH Developer Reagent	160 µl	160 µl	160 µl

Table 5. Pipetting summary

ANALYSIS

Calculations

Plot the Standard Curve:

1. Determine the average absorbance of each standard and sample.
2. Subtract the absorbance value of standard H from itself and all other standards and samples to obtain corrected standard or sample measurements (CSM) for each standard and sample.
3. Plot the CSM of each standard as a function of the final concentration of NADPH from Table 4 on page 18. See Figure 5 on page 22 for a typical standard curve.

Calculate Total, NADPH, and $NADP^+$ Concentration:

1. Calculate the total, NADPH, and/or $NADP^+$ concentrations of the samples using the equation obtained from the linear regression of the standard curve substituting the CSM for each sample.

$$[\text{Total}]\ (\mu M) = \frac{[CSM_{\text{non-heated}} - (\text{y-intercept})]}{\text{Slope}} \times \text{Sample dilution}$$

$$[\text{NADPH}]\ (\mu M) = \frac{[CSM_{\text{heated}} - (\text{y-intercept})]}{\text{Slope}} \times \text{Sample dilution}$$

$$[NADP^+]\ (\mu M) = [\text{Total}] - [\text{NADPH}]$$

2. Determine the NADPH/$NADP^+$ ratio by dividing the concentration of NADPH by that of $NADP^+$.

Performance Characteristics

Sensitivity:

The assay range is 0-1.5 µM.

The lower limit of detection (LLOD) is defined as a concentration two standard deviations higher than the mean zero value. The LLOD for NADPH in this assay is 0.02 µM.

The lower limit of quantification (LLOQ) is the lowest standard concentration in which the mean signal value - 1.64 X S.D is higher than the signal + 1.64 x S.D. of the zero standard. The LLOQ of this assay is 0.125 µM.

Precision:

When a series of ten A549 and eleven HEK293T cell extract measurements were performed on the same day, the intra-assay coefficients of variation were 1.4 and 2.9%, respectively. When a series of six A549 and five HEK293T cell extract measurements were performed on different days under the same experimental conditions, the inter-assay coefficients of variation were both 7%.

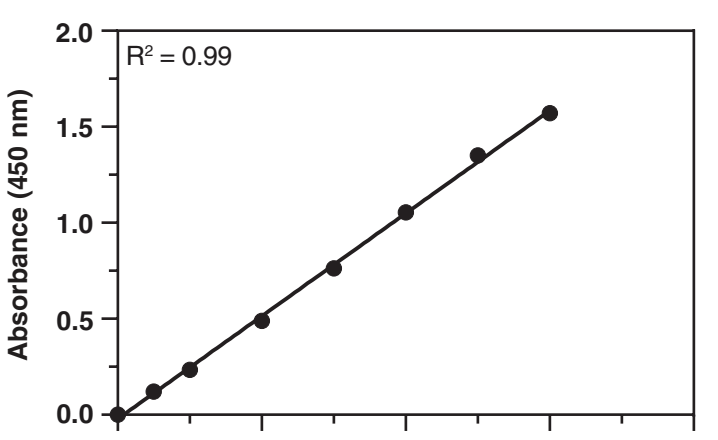

Figure 5. Standard curve

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 absorbance was detected above the zero standard in the sample	A. Sample was too dilute B. The amount of target(s) in the sample is below the LLOD	A. Re-assay the sample using a lower dilution B. Repeat extraction using a lower volume of Extraction Buffer N to concentrate the sample
Absorbance in the sample wells are above the absorbance of the highest standard	Sample was too concentrated	Re-assay the sample using a higher dilution
$NADP^+$ value is higher than total	A. Evaporation during heating may concentrate sample B. Dilution/calculation error	A. Heat sample in a sealed vessel and briefly centrifuge down any condensation B. Recalculate values using the correct sample dilution factor

Assay Summary

Item No.	Reagent	Procedure
400462	Extraction Buffer N	Thaw at room temperature
400463	NADPH Reaction Buffer	Thaw at room temperature
400466	Developer W1	Thaw at room temperature
400781	Developer W2	Thaw at room temperature
400467	NADPH Enzyme	Reconstitute one vial with 1.5 ml NADPH Reaction Buffer
400464	NADPH Standard	Reconstitute the vial with 1 ml of pure water to make a 200 µM solution; dilute to 5 µM with Extraction Buffer N prior to preparing standards (See Table 4 on page 18)
-	NADPH Developer	Combine 16.68 ml NADPH Reaction Buffer, 1.2 ml NADPH Converter Enzyme, 1.2 ml Developer W1 and 120 µl Developer W2

Table 6. Reagent preparation summary for full 96-well plate

Procedure	Standard Wells	Sample Wells
Add standards	40 µl	--
Add samples	--	40 µl
Add NADPH Developer	160 µl	160 µl
Cover and incubate for 60 minutes at room temperature. Read absorbance at 450 nm.		

Table 7. Assay summary

References

1. Jackson, J.B. *Biochim. Biophys. Acta* **1817(10)**, 1839-1846 (2012).
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6. Barcia-Vieitez, R. and Ramos-Martinez, J.I. *IUBMB Life* **66(11)**, 775-779 (2014).
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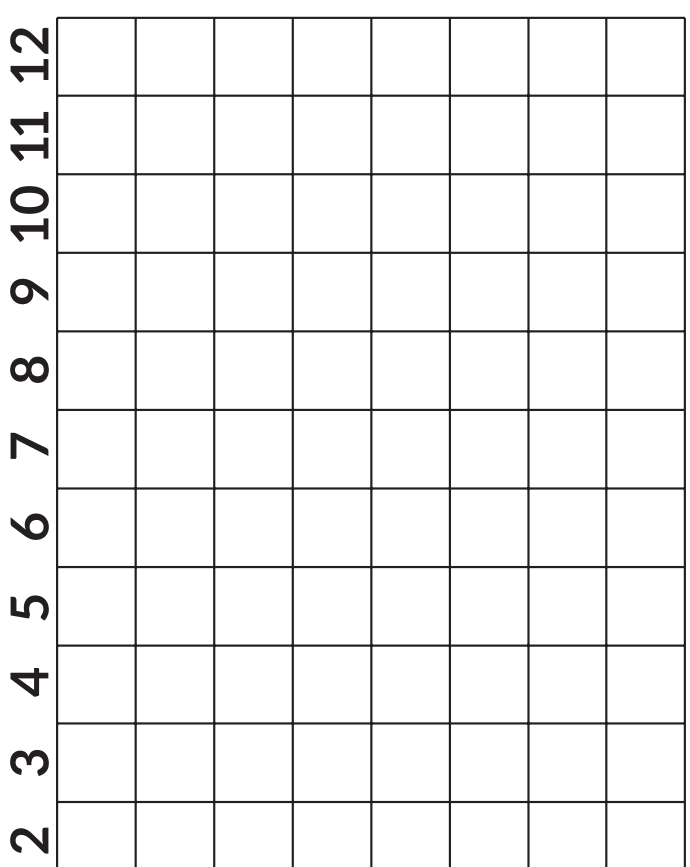

NOTES

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.