PAD4 Autoantibody High-Sensitivity ELISA Kit

Item No. 502865

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 wells Quantity/Size	Storage
400931	Goat Anti-Human Ig (H+L)/HRP Conjugate	1 vial/1.5 ml	4°C
400848	PAD4 Precoated 96-Well Strip Plate	1 plate	4°C
401007	Anti-PAD4 (human) ELISA Standard	1 vial	4°C
401703	Immunoassay Buffer C Concentrate (10X)	1 vial/10 ml	4°C
400062	Wash Buffer Concentrate (400X)	1 vial/5 ml	RT
400035	Polysorbate 20	1 vial/3 ml	RT
400074	TMB Substrate Solution	1 vial/12 ml	4°C
10011355	HRP Stop Solution	1 vial/12 ml	RT
400012	96-Well Cover Sheet	3 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 Anti-PAD4 (human) ELISA Standard was affinity-purified from human plasma. Prior to processing at Cayman Chemical facilities, this standard screened negative for HIV and hepatitis virus. However, we recommend it still be considered potentially infectious. Wear disposable gloves while handling this reagent and thoroughly wash hands afterwards.

The reagents in this kit have been tested and formulated to work exclusively with Cayman's PAD4 Autoantibody High-Sensitivity ELISA Kit. This kit may not perform as described if any reagent or procedure is replaced or modified.

The stop solution provided with this kit is an acid solution. Please wear appropriate personal protection equipment (*e.g.*, safety glasses, gloves, and lab coat) when using this material.

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 ultrapure water, with a resistivity of 18.2 MΩ.cm and total organic carbon (TOC) levels of <10 ppb, 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. Materials used for **Sample Preparation** (see page 10)

INTRODUCTION

Background

Protein arginine deiminase 4 (PAD4) catalyzes the conversion of arginine residues to citrulline within cellular protein substrates, resulting in the loss of a positive charge, which can alter protein structure and/or function.[1] It is expressed in immune cells, including neutrophils and monocytes, as well as in synovial and other tissues.[2] PAD4 has a key role in NETosis, a lytic form of cell death characterized by the release of neutrophil extracellular traps (NETs), which contain proteins, including PAD4, that can act as autoantigens and initiate the formation of anti-citrullinated protein antibodies (ACPAs).[1,3]

PAD4 and PAD4/PAD3 autoantibodies bind to PAD4 and affect its citrullination activity.[2] Patient-derived PAD4 autoantibodies bind to Pad4 on the surface of monocytes, induce chemokine production, and increase disease severity in a mouse model of rheumatoid arthritis (RA).[4] *PADI4* SNPs, including G55S, V82A, and G112A, are associated with RA, and the levels of serum PAD4 autoantibodies from patients with RA are positively associated with disease severity.[5,6]

About This Assay

Cayman's PAD4 Autoantibody High-Sensitivity ELISA Kit is an immunometric assay that can be used to measure IgG, IgM, or IgA isotypes of PAD4 autoantibodies in human plasma and serum without prior sample purification.

The levels of PAD4 autoantibodies in samples can be estimated using a standard curve. Please note, these levels are only approximate as polyclonal antibodies present in the sample will have different affinities to the recombinant PAD4 compared to the purified polyclonal antibody standard supplied in this kit. The standard curve spans the range of 0-125 U/ml, with a lower limit of detection (LLOD) of 0.2 U/ml. One unit of anti–PAD4 Ig is defined based on immunoreactivity and is calibrated to an internal reference standard.

Definition of Key Terms

Standard Curve: a plot of the absorbance values *versus* concentration of a series of wells containing various known amounts of analyte.

Lower Limit of Detection (LLOD): the smallest measure that can be detected with reasonable certainty for a given analytical procedure. The LLOD is defined as a concentration two standard deviations higher than the mean zero value.

Lower Limit of Quantification (LLOQ): the lowest standard concentration in which absorbance (450 nm) – (1.64 x S.D.) is higher than the mean zero value of absorbance (450 nm) + (1.64 x S.D.).

Principle Of This Assay

Each well of the microwell plate supplied in the kit has been coated with recombinant human PAD4. Autoantibodies specific for PAD4, if present in the biological fluid sample, will bind to the immobilized PAD4. A detection antibody recognizing human immunoglobulins (Goat Anti-Human Ig (H+L)) is added to the well. The Goat Anti-Human Ig (H+L) antibody is labeled with HRP, allowing quantitation of the autoantibody. Addition of the HRP substrate 3,3',5,5'-tetramethylbenzidine (TMB), followed by HRP Stop Solution produces a yellow-colored product which can be measured spectrophotometrically. The intensity of the color is directly proportional to the amount of bound Goat Anti-Human Ig (H+L)/HRP Conjugate, which is proportional to the concentration of the PAD4 autoantibody.

Absorbance $\propto$ [Goat anti-human Ig/HRP] $\propto$ [PAD4 autoantibody]

A schematic of this process is shown in Figure 1, below.

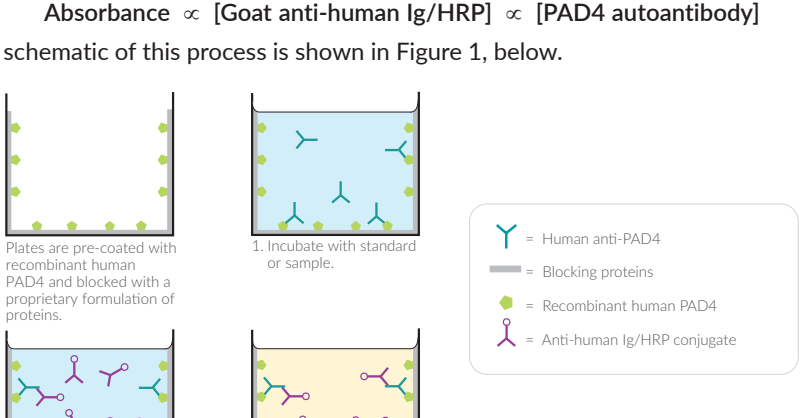


Figure 1. Schematic of the ELISA

PRE-ASSAY PREPARATION

Buffer Preparation

Store all diluted buffers at 4°C; they will be stable for at least two months. *NOTE: It is normal for the concentrated buffer to contain crystalline salts after storage at 4°C. These will completely dissolve upon dilution with ultrapure water.*

1. **Assay Buffer Preparation**

   Dilute the contents of the Immunoassay Buffer C Concentrate (10X) (Item No. 401703) vial with 90 ml of ultrapure water. Be certain to rinse the vial to remove any salts that may have precipitated.

2. **Wash Buffer (1X) Preparation**

   Dilute the contents of the Wash Buffer Concentrate (400X) (Item No. 400062) vial with ultrapure water to a total volume of 2 L and add 1 ml of Polysorbate 20 (Item No. 400035). *NOTE: Polysorbate 20 is a viscous liquid and cannot be measured by a regular pipette. A positive displacement pipette or a syringe should be used to deliver small quantities accurately.*

Sample Preparation

In general, human serum or plasma can be used directly in the assay following a dilution of at least 1:800 with Assay Buffer, in order to fall within the range of the standard curve.

Testing for Interference

This assay has been validated in plasma and serum. Other sample matrices could cause interference and may require dilution. It is recommended to test for interference before embarking on a large number of measurements. To test for interference, dilute one or two test samples with Assay Buffer to obtain several different dilutions for each sample. The dilution factor where the change in the final calculated PAD4 autoantibody concentration is consistent, differing by 20% or less than the previous dilution, is the minimum required dilution for that particular sample type.

Sample Matrix Properties

Parallelism

To assess parallelism, human plasma and serum samples were serially diluted with Assay Buffer and evaluated using the PAD4 Autoantibody High-Sensitivity ELISA Kit. Measured concentrations were plotted as a function of sample dilution. The results are shown below.

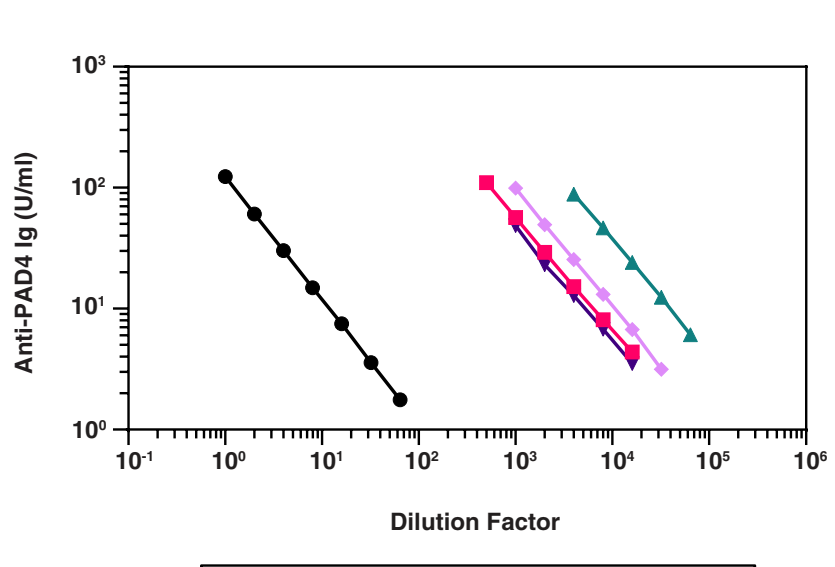


Figure 2. Parallelism of various matrices in the PAD4 Autoantibody High-Sensitivity ELISA Kit

Recovery

Two human EDTA plasma samples were mixed at the ratios indicated below, diluted with Assay Buffer, and analyzed using the PAD4 Autoantibody High-Sensitivity ELISA Kit. The results are shown in the table below. The expected concentration is calculated from the indicated ratios.

% Sample 1	% Sample 2	Expected concentration (kU/ml)	Measured Concentration (kU/ml)	% Recovery
100	0	--	55.6	--
80	20	69.1	68.8	100
60	40	82.6	82.8	100
40	60	96.0	104	108
20	80	110	113	103
0	100	--	123.0	--

Table 1. Recovery of PAD4 autoantibody in a mixed sample

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

Anti-PAD4 (human) ELISA Standard

Reconstitute the lyophilized Anti-PAD4 (human) ELISA Standard (Item No. 401007) with 2.0 ml of Assay Buffer and vortex briefly. The solution will be slightly opaque. The concentration of this solution is 125 U/ml. The reconstituted standard will be stable for 2 weeks at 4°C. Enough standard is provided to produce four standard curves in duplicate for use on different days, if necessary.

To prepare the standard for use in the ELISA: Obtain eight clean test tubes or plastic microfuge tubes and label them #1 through #8. Aliquot 250 µl of Assay Buffer into tubes #2-8. Transfer 500 µl of the reconstituted standard (125 U/ml) to tube #1. Serially dilute the standard by removing 250 µl from tube #1 and placing into tube #2. Mix gently. Next, remove 250 µl from tube #2 and place into tube #3; mix gently. Repeat this process for tubes #4-7. Do not add any standard to tube #8. This tube is the zero standard.

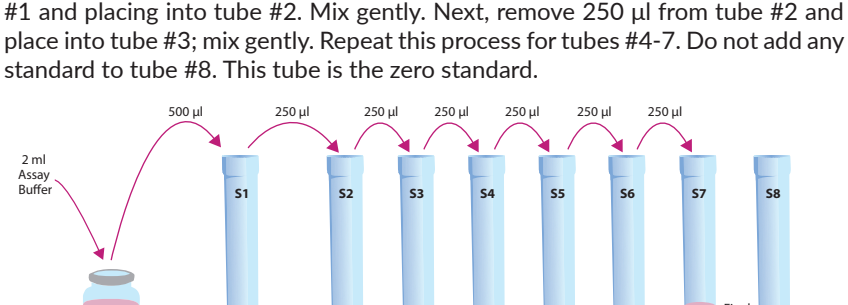


Figure 3. Preparation of the anti-PAD4 standards

Goat Anti-Human Ig (H+L)/HRP Conjugate

Goat Anti-Human Ig (H+L)/HRP Conjugate (Item No. 400931) is supplied as a concentrated (10X) stock solution of goat anti-human Ig polyclonal antibody conjugated to HRP. For a full plate, dilute 1.2 ml of the conjugate with 10.8 ml of Assay Buffer; for a half plate, dilute 0.6 ml of the conjugate with 5.4 ml of Assay Buffer to make a 1X working solution. The diluted conjugate will be stable for 3 hours at room temperature. Any undiluted Goat Anti-Human Ig (H+L)/HRP Conjugate can be stored at 4°C.

Plate Set Up

The 96-well plate(s) included with this kit is supplied ready to use. It is not necessary to rinse the plate(s) prior to adding the reagents. *NOTE: If you do not need to use all of the strips at once, place the unused strips back in the plate packet and store at 4°C. Be sure the packet is sealed with the desiccant inside.*

Each plate or set of strips must contain an eight-point standard curve run in duplicate. Each sample should be assayed at a minimum of two dilutions, and each dilution should be assayed at least in duplicate.

A suggested plate format is shown in Figure 4, below. The user may vary the location and type of wells present as necessary for each particular experiment. We suggest you record the contents of each well on the template sheet provided (see page 26).

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	1	1	9	9	17	17	25	25	33	33
B	S2	S2	2	2	10	10	18	18	26	26	34	34
C	S3	S3	3	3	11	11	19	19	27	27	35	35
D	S4	S4	4	4	12	12	20	20	28	28	36	36
E	S5	S5	5	5	13	13	21	21	29	29	37	37
F	S6	S6	6	6	14	14	22	22	30	30	38	38
G	S7	S7	7	7	15	15	23	23	31	31	39	39
H	S8	S8	8	8	16	16	24	24	32	32	40	40

S1-S8 = Standard Wells
1-40 = Sample Wells

Figure 4. Sample plate format

Performing the Assay

Pipetting Hints

- Use different tips to pipette each reagent.
- 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.

Addition of Standards and Samples and First Incubation

1. Add 100 µl of the standards or diluted samples to the appropriate wells on the plate.
2. Cover the plate with a 96-Well Cover Sheet (Item No. 400012). Incubate for two hours at room temperature on an orbital shaker.

Addition of Goat Anti-Human Ig (H+L)/HRP Conjugate and Second Incubation

1. Empty the wells and rinse five times with ~300 µl Wash Buffer (1X). After the last wash, gently tap the inverted plate on absorbent paper to remove the residual wash buffer.
2. Add 100 µl of the diluted Goat Anti-Human Ig (H+L)/HRP Conjugate to each well of the plate.
3. Cover the plate with a 96-Well Cover Sheet and incubate for one hour at room temperature on an orbital shaker.

Development of the Plate

1. Empty the wells and rinse five times with ~300 µl Wash Buffer (1X). After the last wash, gently tap the inverted plate on absorbent paper to remove the residual wash buffer.
2. Add 100 µl of TMB Substrate Solution (Item No. 400074) to each well of the plate.
3. Cover the plate with a 96-Well Cover Sheet. Optimum development is obtained by using an orbital shaker at room temperature for 10 minutes, protected from light.
4. DO NOT WASH THE PLATE. Add 100 µl of HRP Stop Solution (Item No. 10011355) to each well of the plate. Blue wells should turn yellow and colorless wells should remain colorless. *NOTE: The stop solution in this kit contains an acid. Wear appropriate protection and use caution when handling this solution.*

Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, *etc.*
2. Read the plate at a wavelength of 450 nm.

ANALYSIS

Calculations

Plotting the Standard Curve and Determining the Sample Concentration

Using computer reduction software, plot absorbance (linear y-axis) *versus* concentration (linear x-axis) for standards (S1-S8) and fit the data with a quadratic equation. Using the equation of the line, calculate the concentration in each sample, making sure to correct for any sample dilution.

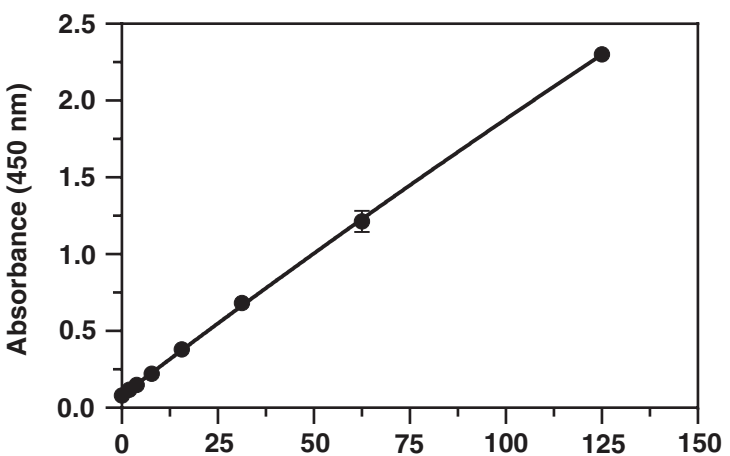
Performance Characteristics

Representative Data

The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve with your experiment. Do not use the data below to determine the values of your samples.

Anti-PAD4 (U/ml)	Absorbance	%CV* Intra-Assay Precision	%CV* Inter-Assay Precision
125	2.301	5.9	2.5
62.5	1.214	5.8	4.6
31.3	0.683	6.4	6.7
15.6	0.380	8.9	7.8
7.8	0.223	7.5	10.3
3.9	0.149	7.4	10.0
2.0	0.117	11.3	29.9**
0.0	0.081	--	--

Table 2. Typical results
*%CV represents the variation in concentration (not absorbance) as determined using a reference standard curve
***Evaluate data in this range with caution*



Assay Range = 0-125 U/ml
Lower Limit of Detection (LLOD) = 0.2 U/ml
Lower Limit of Quantification (LLOQ) = 2.0 U/ml

The standard was diluted in Assay Buffer.

Figure 5. Typical standard curve

Sample Precision:

Intra-assay precision was determined by analyzing 24 replicates of three matrix controls (human plasma) in a single assay.

Plasma Control (kU/ml)	%CV
1,077	3.8
142	5.5
88.4	5.0

Table 3. Sample intra-assay precision

Inter-assay precision was determined by analyzing replicates of three matrix controls (human plasma) in eight separate assays on different days.

Plasma Control (kU/ml)	%CV
1,544	9.6
139	13.2
92.1	16.6

Table 4. Sample inter-assay precision

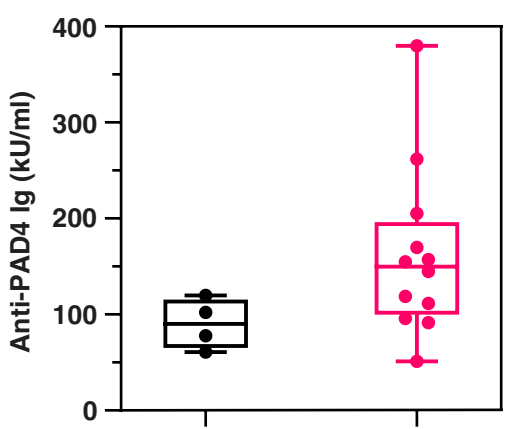


Figure 6. Levels of PAD4 autoantibodies in various human plasma and serum samples. Plasma and serum from both RA and non-RA patients were tested for PAD4 autoantibodies using the PAD4 Autoantibody High-Sensitivity ELISA Kit. Consistent with previous reports indicating that approximately 30% of RA patients exhibit elevated anti–PAD4 autoantibodies, a subset of RA samples in this sample set showed increased levels compared to non-RA controls.[7]

RESOURCES

Troubleshooting

Problem	Possible Causes
Erratic values; dispersion of replicates	A. Trace organic contaminants in the water B. Poor pipetting/technique
Poor development (low signal) of standard curve	A. Plate required more development time B. Standard was diluted incorrectly C. Standard is degraded
Poor development (low signal) of samples	Samples are too dilute
Analyses of two dilutions of a biological sample do not agree (*i.e.*, more than 20% difference)	Interfering substances are present

Assay Summary

Procedure	Standards/Samples
Mix all reagents gently	
Add standards/samples	100 µl
Incubate	Cover the plate and incubate for 2 hours at room temperature on an orbital shaker
Wash	Aspirate wells and wash 5 x ~300 µl with Wash Buffer (1X)
Add Goat Anti-Human Ig (H+L)/HRP Conjugate	100 µl
Incubate	Cover the plate and incubate for 1 hour at room temperature on an orbital shaker
Wash	Aspirate wells and wash 5 x ~300 µl with Wash Buffer (1X)
Add TMB Substrate Solution	100 µl
Develop	Cover the plate and incubate for 10 minutes at room temperature on an orbital shaker, protected from light
Add HRP Stop Solution	100 µl
Read	Read absorbance at 450 nm

References

1. van Beers, J.J.B.C., Zendman, A.J.W., Raijmakers, R., *et al.* Peptidylarginine deiminase expression and activity in PAD2 knock-out and PAD4-low mice. *Biochimie* **95(2)**, 299-308 (2013).
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5. Suzuki, A., Yamada, R., Chang, X., *et al.* Functional haplotypes of *PADI4*, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. *Nat. Genet.* **34(4)**, 395-402 (2003).
6. Halvorsen, E.H., Pollmann, S., Gilboe, I.M., *et al.* Serum IgG antibodies to peptidylarginine deiminase 4 in rheumatoid arthritis and associations with disease severity. *Ann. Rheum. Dis.* **67(3)**, 414-417 (2008).
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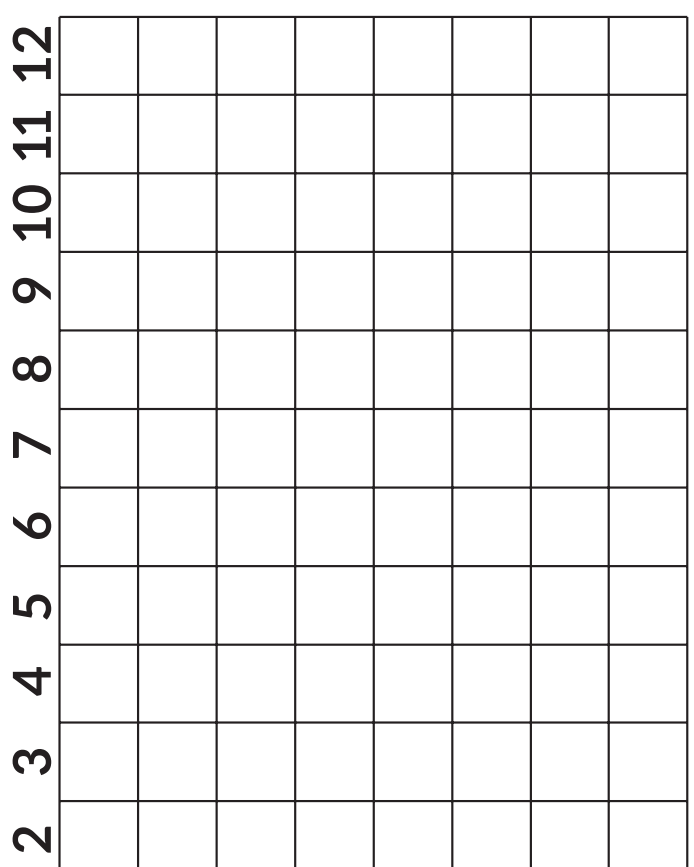

NOTES

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.