PAD2 (human) ELISA Kit

Item No. 501450

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

Item Number	Item	96 wells Quantity/Size
401452	Anti-PAD2 (human) ELISA Strip Plate	1 plate
401451	Anti-PAD2 (human) HRP Conjugate	1 vial/1.5 ml
401454	PAD2 (human) ELISA Standard	1 vial
400060	ELISA Buffer Concentrate (10X)	1 vial/10 ml
400062	Wash Buffer Concentrate (400X)	1 vial/5 ml
400035	Polysorbate 20	1 vial/3 ml
400074	TMB Substrate Solution	1 vial/12 ml
10011355	HRP Stop Solution	1 vial/12 ml
400012	96-Well Cover Sheet	1 cover

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's PAD2 (human) 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

Fax: 734-971-3641

Email: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the batch number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. An orbital microplate shaker set at ~500 rpm.
2. A plate reader capable of measuring absorbance at 450 nm.
3. Adjustable pipettes and a repeating pipettor.
4. A source of pure water; glass distilled or HPLC-grade water is acceptable.

INTRODUCTION

Background

Peptidylarginine deiminases (PADs) are a family of five enzymes that catalyze the conversion of arginine to citrulline in peptides and proteins.[1] PAD2 is the most widely expressed and conserved member across mammalian species, implying it is the ancestral homolog of the PADs.[2] PAD2 may play a role in transcriptional regulation, as it has been shown capable of citrullinating histones, particularly H3 during mammalian reproductive cycles, when it is transcriptionally activated in the nucleus.[3] PAD-dependent citrullination of proteins is implicated in the pathophysiology of rheumatoid arthritis (RA), due to production of anti-citrulline protein antibodies (ACPAs).[1,4] PAD2 activity has been detected in synovial fluid and the synovium of RA patients, with intracellular PAD2 in the synovium correlating with the presence of ACPAs.[5,6] PAD2 activity has also been detected in bronchoalveolar lavage (BAL) fluid.[2] Smoking has been linked to the development of RA due to increased levels of extracellular PAD2 in the lungs relative to non-smokers.[4] PAD enzymes can be produced by cell lines such as HL-60s, a human myeloid leukemia cell line, by inducing differentiation into granulocytes with DMSO or all-*trans* retinoic acid.[7]

About This Assay

Cayman's PAD2 (human) ELISA Kit is a sandwich assay that can be used to measure PAD2 in tissue culture medium, cell lysates, plasma, and serum. The standard curve spans the range of 0.16-10 ng/ml. *NOTE: Rheumatoid factor in samples can give false positives and therefore should be removed from all samples before testing in the assay.*

Principle of the Assay

This sandwich assay is based on a double-antibody 'sandwich' technique. Each well of the microwell plate supplied with the kit has been coated with a monoclonal antibody specific for PAD2 (mouse anti-human PAD2). This antibody will bind any PAD2 introduced into the well. Standards and samples are incubated on the antibody-coated plate, and the plate is then rinsed before addition of an HRP-labeled PAD2 Monoclonal Antibody to detect the captured PAD2. The two antibodies form a 'sandwich' by binding to different locations on the PAD2 protein. The concentration of the analyte is determined by measuring the enzymatic activity of HRP using the chromogenic substrate 3,3',5,5'-tetramethylbenzidine (TMB). After a sufficient period of time, the reaction is stopped with acid, forming a product with a distinct yellow color that can be measured at 450 nm. The intensity of this color, determined spectrophotometrically, is directly proportional to the amount of bound conjugate, which in turn is proportional to the concentration of the PAD2.

$$\text{Absorbance} \propto [\text{Anti-PAD2 HRP}] \propto [\text{PAD2}]$$

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

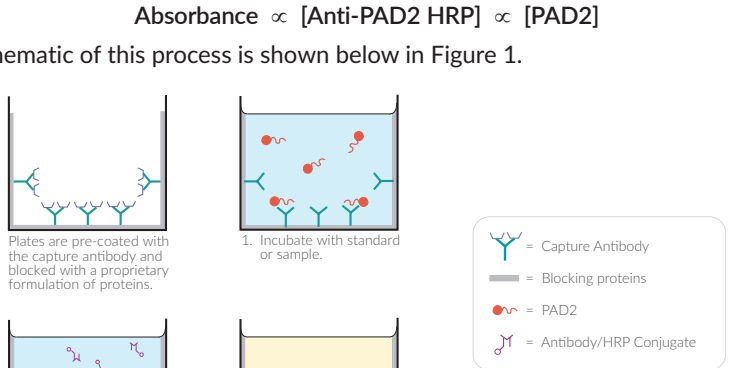


Figure 1. Schematic of the ELISA

Definition of Key Terms

Lower Limit of Quantification (LLOQ): is defined as the lowest standard concentration in which absorbance (450 nm) - (1.64 x S.D.) is higher than the blank value of absorbance (450 nm) + (1.64 x S.D.).

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 point two standard deviations away from the mean zero value.

Dynamic Range: the range in which the analyte is reliably quantifiable.

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

PRE-ASSAY PREPARATION

Buffer Preparation

Store all diluted buffers at 4°C; they should be stable for two months.

1. **ELISA Buffer Preparation**

   Dilute the contents of the vial of ELISA Buffer Concentrate (10X) (Item No. 400060) with 90 ml of water. Be certain to rinse the vial to remove any salts that may have precipitated. *NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with water.*

2. **Wash Buffer Preparation**

   Dilute the contents of the vial of Wash Buffer Concentrate (400X) (Item No. 400062) to a total volume of 2 L with water 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

Sample Collection

All human serum and plasma samples MUST be diluted 1:2 with 1X ELISA Buffer prior to use in this assay. Cell culture media samples can be run undiluted. A minimum volume of 200 µl of each diluted sample is needed to run the samples in duplicate in the assay; for convenience, we recommend that you prepare 250 µl of each diluted sample by combining 125 µl of sample with 125 µl of ELISA Buffer. Dilutions should be performed using polypropylene tubes.

Sample Matrix Properties

Linearity

Human plasma (EDTA), human serum, and DMSO-stimulated cell culture media samples were checked at multiple dilutions using the PAD2 (human) ELISA Kit. Human plasma and human serum were spiked with 10 ng/ml of PAD2 (human) ELISA Standard. The results are shown in the tables below.

Dilution	Concentration (ng/ml)	Dilution Linearity (%)
2	11.5	100
4	11.9	104
8	12.0	104
16	10.7	92

Table 1. Dilutional linearity of spiked human serum in the PAD2 (human) ELISA Kit

Dilution	Concentration (ng/ml)	Dilution Linearity (%)
2	12.8	100
4	11.8	92
8	11.8	93
16	12.5	98

Table 2. Dilutional linearity of spiked human plasma in the PAD2 (human) ELISA Kit

Dilution	Concentration (ng/ml)	Dilution Linearity (%)
1	8.3	100
2	8.2	99
4	8.4	102
8	7.7	93

Table 3. Dilutional linearity of DMSO-stimulated cell culture media in the PAD2 (human) ELISA Kit

Spike and Recovery

Human plasma and human serum were spiked with PAD2, diluted as described in the **Sample Preparation** section and analyzed using the PAD2 (human) ELISA Kit. The results are shown below. The error bars represent standard deviations obtained from multiple dilutions of each sample.

Sample	PAD2 Spiked into Matrix (ng/ml)	% Recovery
Human Serum	10	111.7
	8	128.7
	4	119.2
	2	125.0
Human Plasma	10	117.9
	8	114.1
	4	107.9
	2	101.5

Table 4. Spike and recovery data in human serum and human plasma

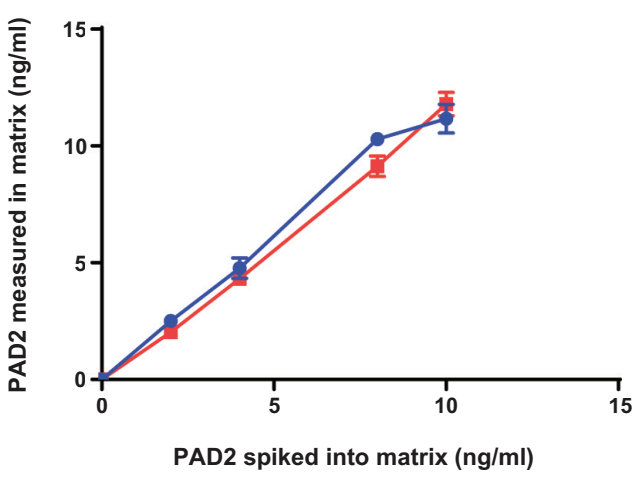


Figure 2. Spike and recovery of PAD2 from human plasma and human serum

Parallelism

To assess parallelism, human plasma, human serum, and DMSO-stimulated cell culture media samples were checked at multiple dilutions by the PAD2 (human) ELISA Kit. Concentrations were plotted as a function of the sample dilution. The results are shown below. Parallelism of the curves demonstrates that the protein binding characteristics are similar enough to allow the accurate determination of protein levels in diluted human plasma, human serum, and DMSO-stimulated cell culture media samples.

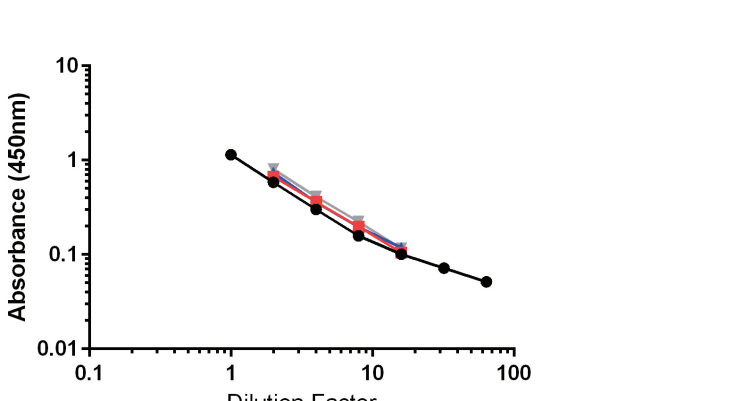


Figure 3. Parallelism of sample matrices in the PAD2 (human) ELISA Kit

Preparation of Assay-Specific Reagents

PAD2 (human) ELISA Standard

Reconstitute the lyophilized PAD2 (human) ELISA Standard (Item No. 401454) with 1 ml of ELISA Buffer. Mix gently. The concentration of this solution (the Bulk Standard) is 1 µg/ml.

To prepare the Standard for use in the ELISA: Dilute 100 µl of bulk Standard (1 µg/ml) into 900 µl ELISA Buffer. This gives a working Standard Solution with a concentration of 100 ng/ml. Obtain eight clean test tubes and label them #1 through #8. Aliquot 900 µl ELISA Buffer to tube #1 and 400 µl ELISA Buffer to tubes #2-8. Transfer 100 µl of the working Standard Solution (100 ng/ml) to tube #1 and mix thoroughly. The concentration of this Standard, the first point on the standard curve, will be 10 ng/ml. Serially dilute the standard by removing 400 µl from tube #1 and placing it in tube #2; mix thoroughly. Repeat this process for tubes #3-7. Do not add any PAD2 to tube #8. This tube is the background control.

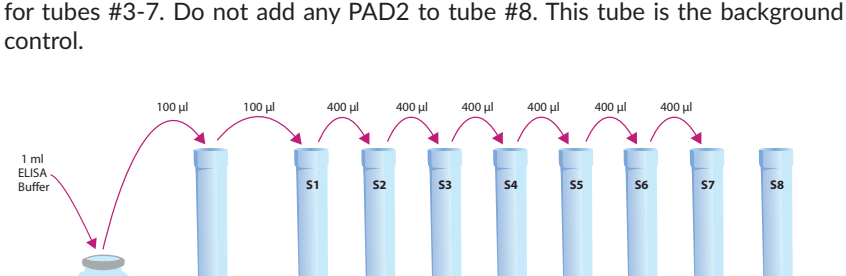


Figure 4. Preparation of the PAD2 Standards

Anti-PAD2 (human) HRP Conjugate

This reagent is supplied as a concentrated (10X) stock solution of anti-PAD2 (human) antibody conjugated to HRP. On the day of the assay, thaw the Anti-PAD2 (human) HRP Conjugate (Item No. 401451). For a full plate, dilute 1.2 ml of HRP Conjugate into 10.8 ml of 1X ELISA Buffer (Item No. 400060); for a half plate, dilute 0.6 ml of HRP Conjugate into 5.4 ml of 1X ELISA Buffer. Do not prepare diluted HRP Conjugate until immediately before use. Store unused Anti-PAD2 (human) HRP Conjugate 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. *NOTE: Each assay must contain this minimum configuration in order to ensure accurate and reproducible results.* Each sample should be assayed at a minimum of two dilutions and each dilution should be assayed in duplicate. For statistical purposes, we recommend assaying samples in triplicate.

A suggested plate format is shown in Figure 5, 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 25).

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	1	1	1	9	9	9	17	17	17	25
B	S2	S2	2	2	2	10	10	10	18	18	18	25
C	S3	S3	3	3	3	11	11	11	19	19	19	25
D	S4	S4	4	4	4	12	12	12	20	20	20	26
E	S5	S5	5	5	5	13	13	13	21	21	21	26
F	S6	S6	6	6	6	14	14	14	22	22	22	26
G	S7	S7	7	7	7	15	15	15	23	23	23	27
H	S8	S8	8	8	8	16	16	16	24	24	24	27

S1-S8 - Standards 1-8
1-27 - Samples

Figure 5. 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(s).

Addition of Standards and Samples and First Incubation

1. Add 100 µl of the PAD2 (human) ELISA Standards or samples into the appropriate wells on the plate. Each sample should be assayed in duplicate, triplicate recommended.
2. Cover the plate with the 96-Well Cover Sheet (Item No. 400012) and incubate for two hours at room temperature on an orbital shaker.

Addition of Anti-PAD2 HRP Conjugate and Second Incubation

1. Empty the wells and rinse four times with 1X Wash Buffer. Each well should be completely filled with 1X Wash Buffer during each wash. Invert the plate between wash steps to empty the fluid from the wells. After the last wash, gently tap the inverted plate on absorbent paper to remove the residual 1X Wash Buffer.
2. Prepare a 1X working solution of the Anti-PAD2 (human) HRP Conjugate (Item No. 401451).
3. Add 100 µl of the Anti-PAD2 (human) HRP Conjugate working solution to each well of the plate, except blank wells.
4. Cover the plate with the 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 four times with 1X Wash Buffer. Each well should be completely filled with 1X Wash Buffer during each wash. Invert the plate between wash steps to empty the fluid from the wells. After the last wash, gently tap the inverted plate on absorbent paper to remove the residual 1X Wash Buffer.
2. Add 100 µl of TMB Substrate Solution (Item No. 400074) to each well of the plate.
3. Cover the plate with the 96-Well Cover Sheet and incubate for 30 minutes at room temperature on an orbital shaker.
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. *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

Many plate readers come with data reduction software that plots data automatically. If your plate reader is capable of analyzing the data, we recommend using a linear fit. Alternatively, a spreadsheet program can be used.

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 linear fit, or alternatively a four-parameter logistic equation. Using the equation of the line, calculate the concentration of PAD2 in each sample.

Performance Characteristics

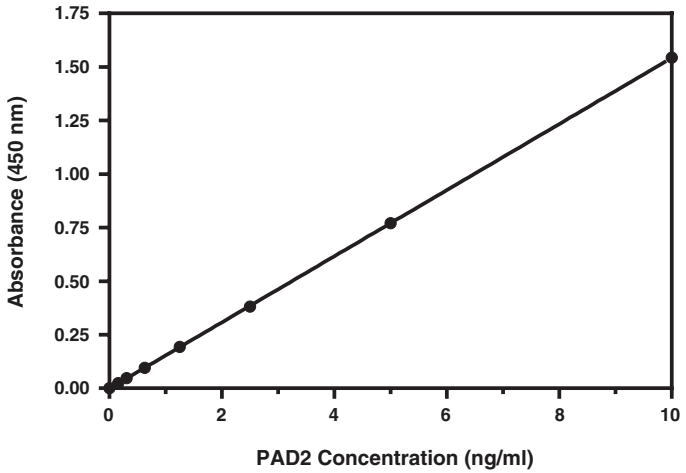
Representative Data

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. Do not use the data below to determine the values of your samples.

Absorbance (450 nm) at 30 minutes

PAD2 Standards (ng/ml)	Absorbance 450 nm	%CV Intra-Assay Precision	%CV Inter-Assay Precision
10	1.543	2.81	13.28
5	0.771	2.02	12.04
2.5	0.381	1.03	10.44
1.25	0.193	1.63	8.80
0.63	0.095	3.63	7.21
0.31	0.047	5.78	8.33
0.16	0.024	12.20	9.34
0	0.001	--	--

Table 5. Typical results



Assay Range = 0.16-10 ng/ml
Dynamic Range = 0.16-10 ng/ml
Sensitivity (defined as LLOQ) = 0.077 ng/ml
Lower Limit of Detection = 0.16 ng/ml

The sensitivity and mid-point were derived from the standard curve shown above. The standard was diluted with ELISA Buffer.

Figure 6. Typical standard curve

Precision:

Intra-assay precision was determined by analyzing 24 replicates of three matrix controls (DMSO stimulated HL-60 cell lysate) a single assay.

Matrix Control (ng/ml)	%CV
9.0	2
5.3	3.2
2.1	2.1

Table 6. Intra-assay precision

Inter-assay precision was determined by analyzing replicates of three matrix controls (DMSO stimulated HL-60 cell lysate) in separate assays spanning across several days .

Matrix Control (ng/ml)	%CV
8.8	5.7
5.4	6.2
2.4	7.3

Table 7. Inter-assay precision

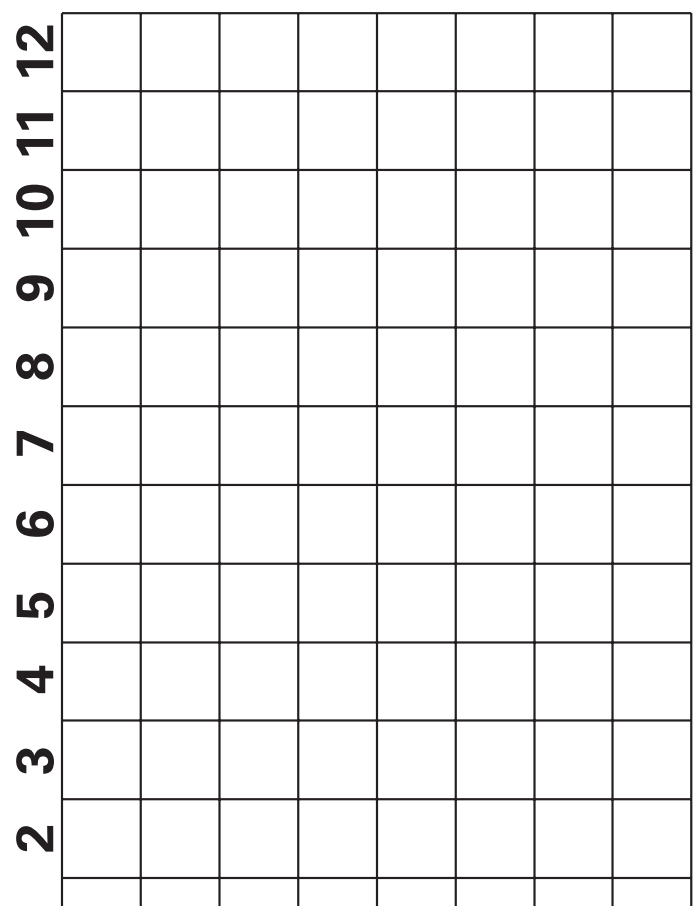
RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Poor development (low signal) of standard curve	A. The standard is degraded B. 10X HRP Conjugate was diluted into 1X ELISA Buffer for too long before use, resulting in inactivation of the HRP C. Standard was diluted incorrectly	Perform assay again with a new aliquot of Standard or HRP Conjugate
Sample concentrations appear inconsistent with literature values	Matrix for samples and standards are different	Use same matrix for all samples and standards

Procedure	Blank	Standards/Samples
Mix all reagents gently	--	--
Add standards/samples to plate	--	100 µl
Seal the plate and tap gently to mix	→	→
Incubate plate for 2 hours at RT, shaking	→	→
Aspirate wells and wash 4 x well volume (~400 µl) with 1X Wash Buffer	→	→
Apply 1X HRP Conjugate Solution	--	100 µl
Incubate for 1 hour at RT, shaking and sealed	→	→
Aspirate wells and wash 4 x well volume (~400 µl) with 1X Wash Buffer	→	→
Apply TMB Substrate Solution	100 µl	100 µl
Incubate 30 min at RT, shaking, sealed, and *protected from light*	→	→
Do Not Wash, apply HRP Stop Solution	100 µl	100 µl
Read absorbance at 450 nm	→	→

Table 8. PAD2 Assay Summary



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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.