HLA-DR (α and β chains) ELISA Kit

Item No. 501810

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GENERAL INFORMATION

Materials Supplied

Item Number	Item Name	96 wells Quantity/Size	Storage Temperature
480131	HLA-DR (α and β chains) ELISA Standard	1 vial	-20°C
480132	Anti-HLA-DR (α and β chains) HRP Conjugate	1 vial/1.5 ml	-20°C
400108	Immunoassay Buffer D Concentrate (5X)	1 vial/10 ml	4°C
400062	Wash Buffer Concentrate (400X)	1 vial/5 ml	RT
400035	Polysorbate 20	1 vial/3 ml	RT
480130	Anti-HLA-DR (α and β chains) ELISA Strip Plate	1 plate	-20°C
400074	TMB Substrate Solution	1 vial/12 ml	4°C
10011355	HRP Stop Solution	1 vial/12 ml	RT
400012	96-Well Cover Sheet	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.

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: 734-971-3640

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 (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 and a repeating pipettor
4. A source of ultrapure water 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 12)

INTRODUCTION

Background

Human leukocyte antigen DR isotope (HLA-DR) is an MHC Class II cell surface receptor heterodimer composed of a 33-35 kDa α chain and an approximately 30 kDa β chain that, together, contain a 13-amino acid peptide-binding cleft.[1,2] HLA-DR is assembled in the endoplasmic reticulum of antigen-presenting cells, such as dendritic cells, macrophages, and B cells.[1] Following assembly, it is transported to the Golgi apparatus and endosome/lysosome compartments for attachment of a peptide fragment that is recognized by $CD4^+$ T cells when presented at the cell surface, leading to activation or suppression of an antibody response.[1,3] HLA-DR polymorphisms are associated with an increased susceptibility for rheumatoid arthritis, systemic lupus erythematosus (SLE), autoimmune hepatitis, and multiple sclerosis.[3-8] However, certain HLA-DR alleles have been identified as protective factors for SLE, rheumatoid arthritis, and multiple sclerosis.[3] The HLA-DR (α and β chains) ELISA Kit recognizes the correctly folded α- and β-chain heterodimeric form of HLA-DR.[9]

About This Assay

Cayman's HLA-DR (α and β chains) ELISA Kit is an immunometric assay (*i.e.* sandwich) that can be used for the quantification of the HLA-DR complex in cell lysates. The standard curve spans the range of 0.63-40 ng/ml, with a limit of quantification of 1.25 ng/ml.

Principle Of This Assay

This immunometric 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 the HLA-DR (α and β chains) complex. This antibody will bind any HLA-DR complex introduced into the well. A second monoclonal antibody conjugated to horseradish peroxidase (HRP), which also recognizes the HLA-DR complex, is added to the well forming a "sandwich". The "sandwich" is immobilized on the plate and the excess reagents are washed away. The concentration of the HLA-DR complex is determined by measuring the enzymatic activity of HRP using the chromogenic substrate 3,3',5,5'-tetramethylbenzidine (TMB). After a sufficient period, the reaction is stopped with acid, forming a product with a distinct yellow color that can be measured at 450 nm. The intensity of the color is directly proportional to the amount of bound antibody-HRP conjugate, which is proportional to the concentration of the HLA-DR (α and β chains) complex.

$$\text{Absorbance} \propto [\text{Anti-HLA-DR HRP}] \propto [\text{HLA-DR complex}]$$

A schematic of this process is shown in Figure 1, on page 9.

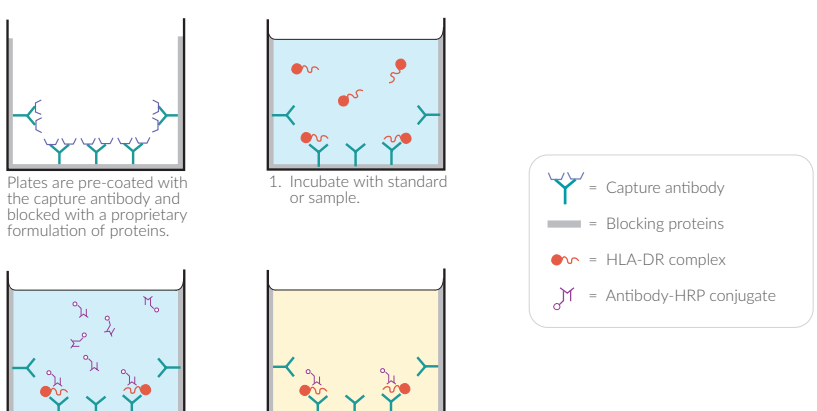


Figure 1. Schematic of the ELISA

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.).

PRE-ASSAY PREPARATION

Buffer Preparation

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

1. **Assay Buffer (1X) Preparation**

   Dilute the contents of one vial of Immunoassay Buffer D (5X) (Item No. 400108) with 40 ml of ultrapure water and add 50 µl of Polysorbate 20 (Item No. 400035). Be certain to rinse the vial to remove any salts that may have precipitated.

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

   Dilute the contents of one vial of Wash Buffer Concentrate (400X) (Item No. 400062) with ultrapure water to a total volume of 2 L and add 1 ml of Polysorbate 20.

Sample Preparation

Sample Collection and Storage

This assay has been demonstrated to work with human cells lysed in Pierce™ IP Lysis Buffer (available from Thermo Fisher Scientific) with added protease inhibitors (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail available from MilliporeSigma). It is recommended to lyse cells on ice for 30 minutes before spinning at 20,000 x g at 4°C for 30 minutes. Collect supernatant and assay immediately or aliquot and store at -20°C. Other sample types, lysis buffers, or concentrated cell lysates may cause interference and require a minimum dilution determined by the end user. Please read this section thoroughly before beginning the assay.

Testing for Interference

To test for interference, dilute one or two test samples to obtain several different dilutions for each sample. The dilution factor where the change in the final calculated HLA-DR 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

Linearity

THP-1 cells (low DR-positive cell line) or Daudi cells (high DR-positive cell line) were lysed at 10 million cells per ml as described in the Sample Preparation section and assayed at multiple dilutions using the HLA-DR (α and β chains) ELISA Kit. The results are shown in the tables below.

Dilution	Concentration (ng/ml)	Dilutional Linearity (%)
10	254	100
20	247	97.3
40	340	134
80	284	112

Table 1. Dilutional linearity of THP-1 cell lysate

Dilution	Concentration (ng/ml)	Dilutional Linearity (%)
200	4,664	100
400	4,398	94.3
800	4,379	93.9
1,600	4,784	103

Table 2. Dilutional linearity of Daudi cell lysate

Spike and Recovery

THP-1 cells (low DR-positive cell line) were lysed as described in the Sample Preparation section, spiked with different amounts of HLA-DR, then diluted and analyzed using the HLA-DR (α and β chains) ELISA Kit. The results are shown below. The error bars represent standard deviations obtained from multiple dilutions of each sample.

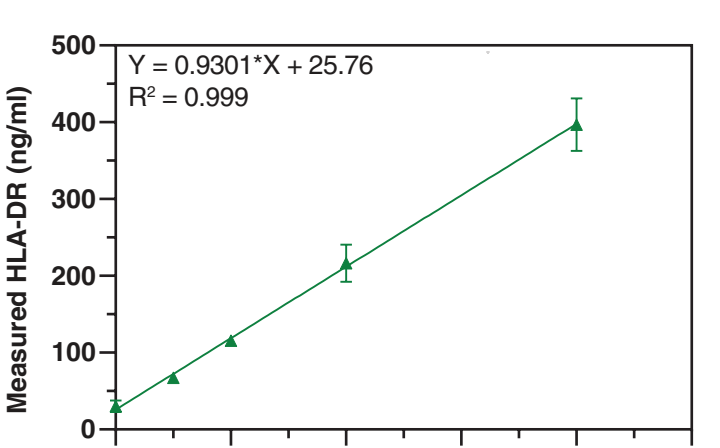


Figure 2. Spike and recovery in THP-1 cell lysate

THP-1 cells (low DR-positive cell line), HL-60 (DR-negative cell line), and Huh7 cells (DR-negative cell line) were lysed at 10 million cells per ml as described in the Sample Preparation section before being spiked with Daudi cell lysate (high DR-positive cell line) prepared in the same manner, diluted, and analyzed using the HLA-DR (α and β chains) ELISA Kit. The results are shown below.

Sample	HLA-DR Spiked (ng/ml)	HLA-DR Measured (ng/ml)	% Recovery
THP-1 Cell Lysate	674	1,020	110
HL-60 Cell Lysate	774	793	94.8
Huh7 Cell Lysate	665	760	113

Table 3. Spike and recovery in THP-1, HL-60, and Huh7 cell lysates spiked with Daudi cell lysate

Parallelism

To assess parallelism, THP-1 (low DR-positive cell line) and Daudi (high DR-positive cell line) cell lysates were assayed at multiple dilutions in the HLA-DR (α and β chains) ELISA Kit. Concentrations were plotted as a function of sample dilution. The results are shown below.

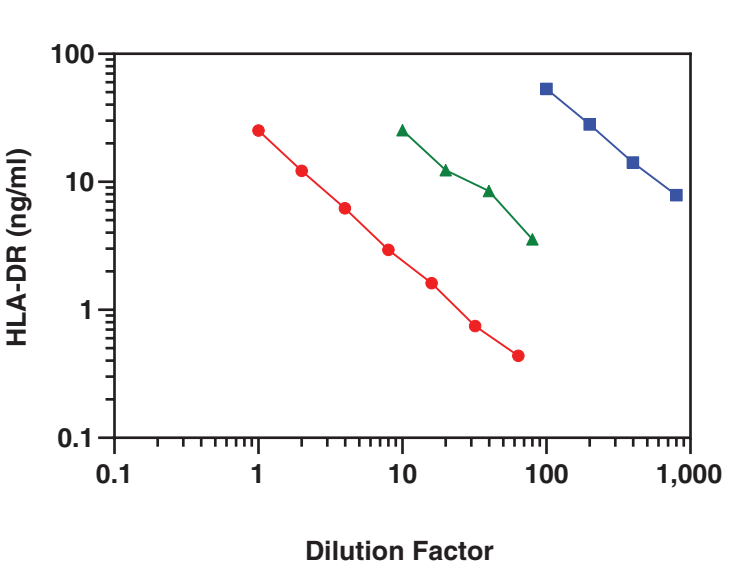


Figure 3. Parallelism of THP-1 and Daudi cell lysates in the HLA-DR (α and β chains) ELISA Kit

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

HLA-DR (α and β chains) ELISA Standard

Reconstitute the lyophilized HLA-DR (α and β chains) ELISA Standard (Item No. 480131) with 0.5 ml of Assay Buffer (1X). Mix gently. The concentration of this solution (the bulk standard) is 400 ng/ml. The reconstituted standard is stable at 4°C for four weeks.

To prepare the standard for use in the ELISA: Obtain eight clean test tubes and label them #1 through #8. Aliquot 900 µl Assay Buffer (1X) into tube #1 and 500 µl Assay Buffer (1X) into tubes #2-8. Transfer 100 µl of the bulk standard (400 ng/ml) to tube #1 and mix gently. Serially dilute the standard by removing 500 µl from tube #1 and placing it in tube #2; mix thoroughly. Repeat this process for tubes #3-7. Do not add any HLA-DR Standard to tube #8. This tube is the background control.

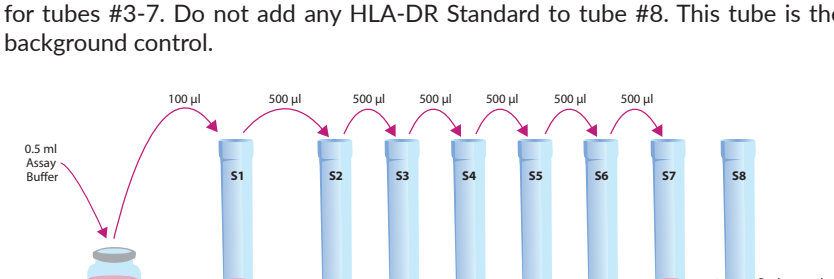


Figure 4. Preparation of the HLA-DR standards

Anti-HLA-DR (α and β chains) HRP Conjugate

Anti-HLA-DR (α and β chains) HRP Conjugate (Item No. 480132) is supplied as a concentrated (10X) stock solution of Anti-HLA-DR antibody conjugated to HRP. On the day of the assay, thaw the antibody-HRP conjugate at room temperature.

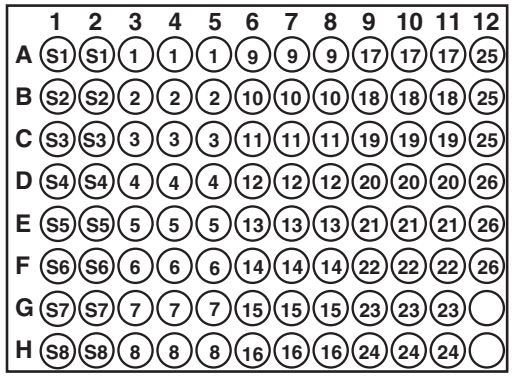
For a full plate, dilute 1.2 ml of the antibody-HRP conjugate into 10.8 ml of Assay Buffer (1X); for a half plate, dilute 0.6 ml of the antibody-HRP conjugate into 5.4 ml of Assay Buffer (1X) to make a 1X working solution. Do not prepare diluted antibody-HRP conjugate until immediately before use. Discard any unused antibody-HRP conjugate (1X). Store Anti-HLA-DR (α and β chains) HRP-Conjugate (10X) stock solution 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 the strips at once, place the unused strips back in the plate packet and store at -20°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 recording the contents of each well on the template sheet provided (see page 29).



S1-S8 - Standards 1-8
1-26 - 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.

Addition of Standards and Samples and First Incubation

1. Pipette 100 µl of the HLA-DR 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), tap gently to mix, and incubate for two hours at room temperature on an orbital shaker.

Addition of Anti-HLA-DR-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. Prepare a 1X working solution of the Anti-HLA-DR-HRP Conjugate as described in the Preparation of Assay-Specific Reagents section.
3. Add 100 µl of the Anti-HLA-DR-HRP Conjugate (1X) working solution to each well of the plate.
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 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 the 96-Well Cover Sheet. Optimum development is obtained by using an orbital shaker at room temperature for 30 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

Many plate readers come with data reduction software that plot data automatically. Alternatively, a spreadsheet program can be used.

Calculations

Plot the Standard Curve and Determine 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 fit. Using the equation of the line, calculate the concentration of HLA-DR complex in each sample, making sure to correct for any sample dilution.

Performance Characteristics

Representative Data

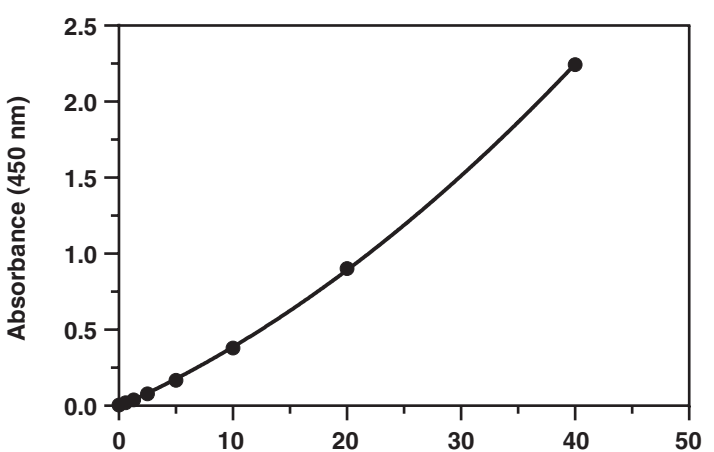
The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve. Do not use the data below to determine the values of your samples.

HLA-DR Standards (ng/ml)	Blank-corrected Absorbance	%CV* Intra-assay Precision	%CV* Inter-assay Precision
40	2.244	3.2	3.6
20	0.902	2.9	4.5
10	0.379	4.2	5.0
5	0.168	5.3	4.0
2.5	0.079	6.6	7.3
1.25	0.038	12.7	14.5
0.625	0.020	24.0**	23.4**
0	0.005	--	--

Table 4. 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 = 0.63-40 ng/ml
Lower Limit of Detection (LLOD) = 0.57 ng/ml
Lower Limit of Quantification (LLOQ) = 1.25 ng/ml

The LLOQ was calculated from the standard curve shown above.
The standard was diluted in Assay Buffer (1X).

Figure 6. Typical standard curve

Precision:

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

Matrix Control (ng/ml)	%CV
32.3	3.6
20.8	2.5
5.8	3.9

Table 5. Intra-assay precision

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

Matrix Control (ng/ml)	%CV
34.5	5.1
25.4	5.6
8.2	6.0

Table 6. Inter-assay precision

RESOURCES

Troubleshooting

Problem	Possible Causes
Erratic values; dispersion of duplicates	A. Trace organic contaminants in the water source B. Poor pipetting/technique
High backgound wells (>0.150 O.D.)	A. Poor washing; ensure proper washing is used B. Exposure of background wells to standards or samples
Poor development (low signal) of standard curve	A. Trace organic contaminants in the water source B. Dilution error in preparing reagents
Poor development (low signal) of samples	A. Insufficient lysis of cells; normalize to protein content of cell lysate B. Specific cell type is not HLA-DR positive
Analyses of two dilutions of a biological sample do not agree (*i.e.*, more than 20% difference)	Interfering substances are present; determine minimal dilution for that sample type

References

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2. Jardetzky, T.S., Brown, J.H., Gorga, J.C., *et al.* Crystallographic analysis of endogenous peptides associated with HLA-DR1 suggests a common, polyproline II-like conformation for bound peptides. *Proc. Natl. Acad. Sci. U.S.A.* **93(2)**, 734-738 (1996).
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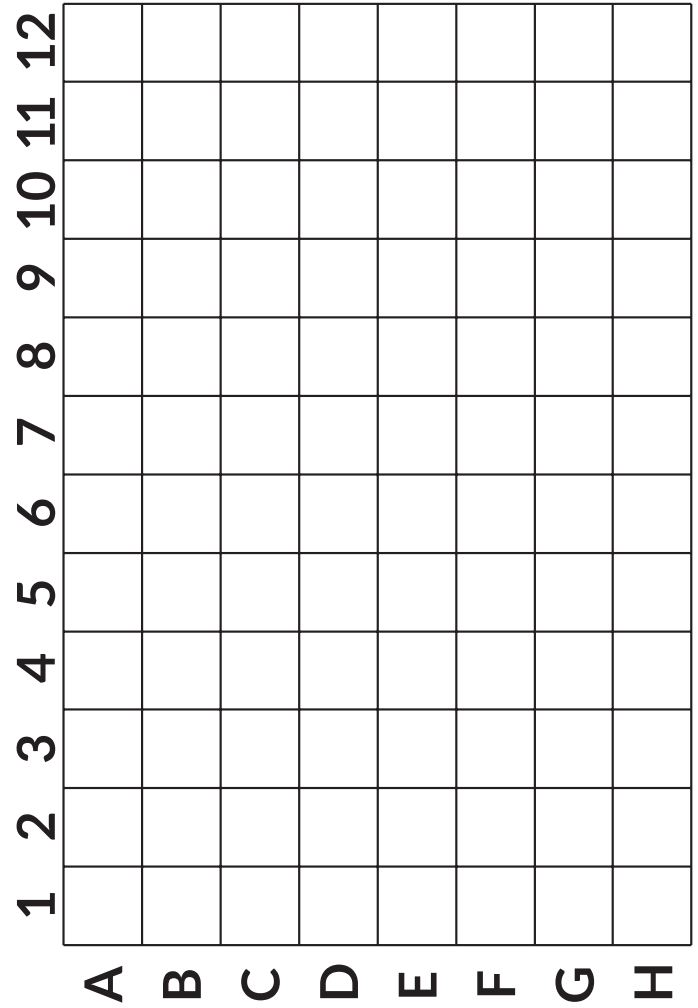
6. van Lith, M., McEwen-Smith, R.M., and Benham, A.M. HLA-DP, HLA-DQ, and HLA-DR have different requirements for invariant chain and HLA-DM. *J. Biol. Chem.* **285(52)**, 40800-40808 (2010).
7. Oliveira, L.C., Porta, G., Marin, M.L.C., *et al.* Autoimmune hepatitis, HLA and extended haplotypes. *Autoimmun. Rev.* **10(4)**, 189-193 (2011).
8. Luckey, D., Bastakoty, D., and Mangalam, A.K. Role of HLA class II genes in susceptibility and resistance to multiple sclerosis: Studies using HLA transgenic mice. *J. Autoimmun.* **37(2)**, 122-128 (201).
9. Stockel, J., Meinl, E., Hahnel, C., *et al.* Refolding of human class II major histocompatibility complex molecules isolated from *Escherichia coli*. Assembly of peptide-free heterodimers and increased refolding-yield in the presence of antigenic peptide. *J. Biol. Chem.* **269(47)**, 29571-29578 (1994).

Procedure	TMB Blank	Standards/Samples
Mix all reagents gently	--	
Add standards/samples to plate	--	100 µl
Seal	Seal the plate and tap gently to mix	
Incubate	Incubate plate for 2 hours at RT, shaking and sealed	
Aspirate	Aspirate wells and wash 5 x ~300 µl with Wash Buffer (1X)	
Apply antibody-HRP conjugate solution (1X)	--	100 µl
Incubate	Incubate plate for 1 hour at RT, shaking and sealed	
Aspirate	Aspirate wells and wash 5 x ~300 µl with Wash Buffer (1X)	
Apply TMB Substrate Solution	100 µl	100 µl
Seal	Seal plate and incubate for 30 minutes at room temperature on orbital shaker, protected from light	
Apply HRP Stop Solution, do not wash	100 µl	100 µl
Read	Read absorbance at 450 nm	

Table 7. Assay Summary



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.