Development of a Standardized HLA-E Assay Procedure

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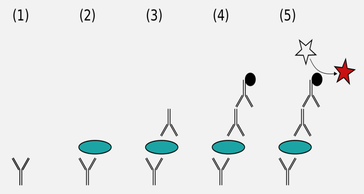
Abstract

Human leukocyte antigen-E (HLA-E) is a nonclassical HLA class I molecule that canonically binds peptides derived from the leader sequence of classical HLA class I proteins. The immunological and clinical relevance of HLA-E is poorly investigated and our aim was to provide a standardized method for their quantitation. The Enzyme-linked Immunosorbent Assay (ELISA) is a popular diagnostic tool to detect or quantify a protein in a liquid sample. The test developed for this project is the sandwich ELISA method, a variant which involves two antibodies with specificity for the HLA protein. The sandwich ELISA is preferable for our purposes because samples need not be purified before testing. However, low signals, low sensitivity, high background signal (due to a high amount of enzyme conjugate), poor standard curve linearity, and an unpredictabe dynamic range are common without optimization of the test parameters. Here, we created a standard operating procedure (SOP) for the quantitative determination of sHLA-E molecules in fluidic probes. Three major parameters of the assay were identified and independently evaluated:
Parameter 1: the optimal coating concentration for the capture antibody was found to be 20 $^{\mu g}/_{ml}$. Parameter 2: sHLA concentrations of the standard covering the dynamic range of the assay was found to be optimal from 0.1 to 10 $^{\mu g}/_{ml}$. Parameter 3: the biotinylated detection antibody dilution factor was found to be most appropriate at a 500-fold dilution (720 $^{ng}/_{ml}$).

Introduction

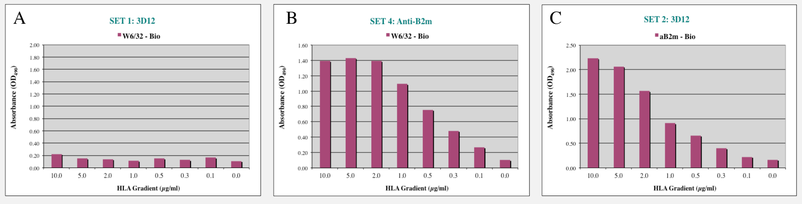
The focus of this study was the development of an assay to quantify HLA-E Class I molecules in solution. HLA-E is a non-classical heterodimer consisting of an α-heavy chain and a β2-microglobulin light chain. The molecule has a canonical binding motif for peptides, that is consistent with other types of HLA. As well, the characteristic (non-classical) low variability of the HLA-E molecules means that there is very little fluctuation in the proteins polymorphism. For this reason, there are currently only two known dominant forms of the molecule: HLA-E*01:01 and HLA-E*01:03. We are focusing on HLA-E because its immunological and clinical relevance is poorly investigated. Its potential to act as a disease marker, particularly if increased serum levels of soluble HLA-E correlates with disease activity is worth investigating. Our goal for this project was to establish a standardized method by which to quantitatively detect sHLA-E to be able to investigate sHLA-E quantities in blood serum of a diseased patient vs. a normal individual.

Sandwich ELISA Procedure Overview:



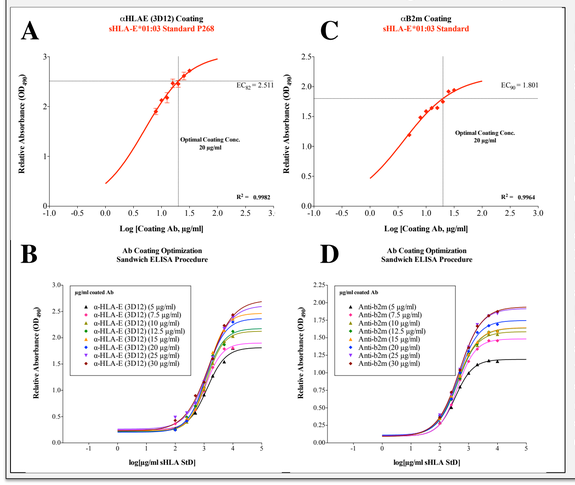
1. Surface is coated with an antibody
   - Wash (Not Shown)
2. HLA-E Samples applied with 2% Casein diluent
   - Wash
3. Detector Antibody added
   - Wash
4. Enzyme-linked secondary antibody added
   - Wash
5. Substrate added, which is converted to a detectable form by an HRP enzyme

Evidence of Steric Hindrance



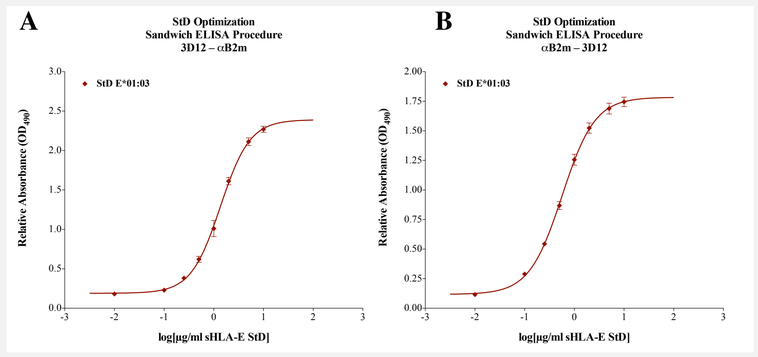
With the W6/32^{Bio} on 3D12 combination (A), we observed that no standard curve was produced and only low background readings were recorded. However, because of a successful signal development generated from the W6/32^{Bio} on α-β2M (B) and α-β2M^{Bio} on 3D12 (C) combinations, we concluded that both biotinylated W6/32 and non-modified 3D12 are capable of binding and signaling when paired separately. This result suggests an instance of steric hindrance (A) between the coating antibody 3D12 and the detector antibody W6/32 who both compete for an HLA-E binding site in close proximity to each other. To be more specific, when the HLA-E standard binds to the 3D12 coating antibody, it is taking up space around the binding area, thus preventing the W6/32 antibody from binding to that space.

Results:
PARAMETER 1: Coating Antibody Concentration



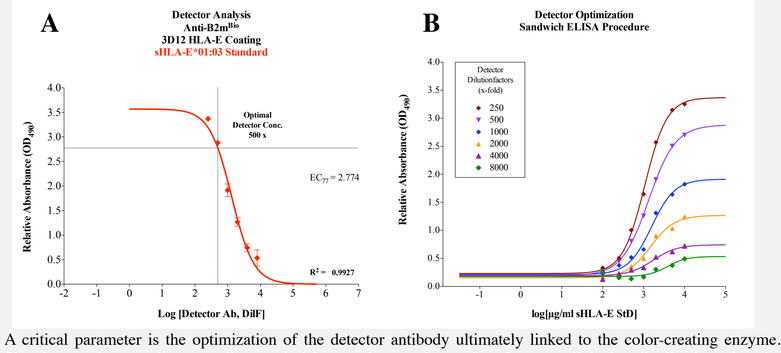
In order to capture sufficient sample HLA molecules for sensitive analysis, the coating of the capture antibody needed to be maximized. Various concentrations were tested with the goal of not over-saturating the coating to prevent artificial binding effects or steric hindrance. (A) A sigmoidal dose-response curve (7 best-fit values) was generated as a function of log of coating antibody concentration. An optimal value of 20 µg/ml for 3D12 was selected, which is at 82% maximal signal with an expected signal of 2.511. (B) Individual standard titration curves at different coatings were shown. As an alternative, the α-β2m antibody was also optimized for HLA-E capturing (C), showing comparable results, however, with a lesser capacity of binding at the lower end (D).

Results:
PARAMETER 2: HLA Standard Dynamic Range Determination



Because quantitative measurements can only be accurately performed when operating within the linear range of the assay, it was necessary to first create an acceptable standard curve of the HLA-E standard. Twelve standard concentrations from 0.1 to 10 $^{\mu g}/_{ml}$ were selected to create an optimal standard curve with adequate spacing between points. The two alternative assays (A) 3D12 – anti-β2m an (B) anti-β2m – 3D12 achieved similar results confirming the suitability of the HLA-E standard in creating a linear range of around 2 Logs.

Results
PARAMETER 3: Detection Antibody Dilution-factor



A critical parameter is the optimization of the detector antibody ultimately linked to the color-creating enzyme. Signal saturation can distort readings and obfuscate the actual quantity of enzyme-linked antibodies bound to the antigen. (A) A sigmoidal dose-response curve (6 best-fit values) plotted as a function of log detection antibody dilution factor indicates that it takes a 500-fold dilution of the detection antibody to reach 77% of the maximal signal at OD=2.774, a value which is well below saturation. (B) Individual standard titrations curves at different detector concentrations show the relative absorbance of the HLA-E standard under the various dilutions of the α-β2M^{Bio} detector antibody.

Method

Coating Antibody Concentration Optimization

A stock of 3D12/anti-β2m coating antibody was serially diluted in Phosphate buffer, pH 7.2. Diluted solutions ranged from 5 to 30 µg/ml. Solutions were distributed to microtiter plates in triplicates with 100 µL per well. The plates were incubated overnight at 4 °C. After the coating step, they were washed and unoccupied binding sites on the plate were blocked by adding 200 µL of 3% BSA to each well. The plates were incubated for 2 hours at RT or overnight at 4°C. Subsequent assay parameters were kept constant.

HLA-E Standard Dynamic Range Optimization and Standard Variation

After coating with optimal coating antibody concentration and blocking, 50 µL of serially diluted HLA-E standard ranging from 0.1 to 10 µg/ml were pipetted in each well. Each diluted solution was distributed in triplicates to the plates. The plates were incubated for 1 hour at RT and washed. Succeeding parameters were kept fixed.

Step	Procedure	Incubation Time	Amount per well
1	Coating	4°C ON	100 µl
	- Wash		
2	Blocking	2 hours RT/4°C ON	200 µl
	- Wash		
3	Test-Samples (3% BSA)	1 hour at RT	50 µl
	- Wash		
4	Detection Antibody	1 hour at RT	100 µl
	- Wash		
5A	Preincubate ABC	30 min at RT	N/A
5B	Detection (ABC)	1 hour at RT	100 µl
	- Wash		
6	Substrate/OPD	20 Min	100 µl
7	Stop Reaction (H_2SO_4)		100 µl
8	Read plates		

Biotinylated Detection Antibody Dilution Factor Optimization

After coating and applying the optimized HLA standard range, 100 µL of serially diluted biotinylated detection antibody in 3% BSA ranging from 250- to 8000-fold were added to each well. Each X-fold solution was distributed to a plate in duplicate. It was incubated at RT for 1 hour. The plate was washed. 100 µL of 30 minutes RT pre-incubated Avidin-biotin complex-horseradish peroxidase (ABC-HRP) in 10 ml 1X PBS, pH 7.4 was added to each well for 1 hour incubation at RT. The plate was washed. Following steps were not altered.

Bw6 reactivity on the HLA-E

	77	78	79	80	81	82	83	Position
E*01:03	N	L	R	T	L	R	G	[Bw6]
B*07:02	S	L	R	N	L	R	G	Bw6
B*73:01	G	L	R	N	L	R	G	Bw6
B*57:01	N	L	R	I	A	L	R	Bw4
B*13:01	N	L	R	T	A	L	R	Bw4
B*27:05	D	L	R	T	L	L	R	Bw4
A*32:01	S	L	R	I	A	L	R	Bw4
A*01:01	N	L	G	T	L	R	G	–
A*02:01	D	L	G	T	L	R	G	–

Based on the mutual exclusivity of HLA-B, some HLA-C, and likewise several HLA-A alleles in expressing the Bw6 epitope (the portion of the antigen recognized by the antibody), a test was conducted to verify whether or not HLA-E also contains this epitope. In order to demonstrate this, a specific Bw6-recognizing antibody was used as the detector antibody. From the test (data not shown), we could conclude that the canonical binding motif for Bw6 represented by HLA alleles B*07:02 and B*73:01 can be extended to HLA-E*01:03 despite the discrepancy at position 80.

Conclusion

The creation of Standard Operating Procedures for Sandwich ELISA tests are quite common in clinical and commercial setups, especially since many laboratories count on the repeatability and accuracy that is inherent to them. The results given here show that, with a small amount of optimization in the steps of the procedure, a quality assay can be generated. To that end, for the detection of sHLA, we found an optimal coating concentration of 20 µg/ml for both coating antibodies tested. The optimal dynamic range for the HLA-E standard was from 0.1 to 10 µg/ml giving a broad spectrum in which quantitative analysis can be performed. In order to prevent the possible problem of saturation of the system, a 500-fold dilution of the detection antibody in combination with a 20 minute substrate incubation time resulted in a maximal signal output of 2.774 OD_{490} for the 3D12/anti-β2m combination, which allows accurate and sensitive measurements of unknown samples.