ELISA Assay Buffer Additives to Solve Matrix Interferences in a Biomarker Assay

Andrew P. Mayer[1], Michael L. Schneider[1], Ronald R. Bowsher[1]
[1]AIT Bioscience, 7840 Innovation Drive, Indianapolis, IN 46278

Purpose

The purpose was to establish a sandwich ELISA optimized for the quantitative determination of human macrophage-colony stimulating factor (M-CSF) from bulk critical reagents. During method development two primary challenges had to be overcome: 1) matrix interference; and 2) replicate imprecision. A step-by-step systematic process to establish a buffer-based assay for the quantification of M-CSF in samples of human K_2EDTA plasma was performed, followed by characterization of the assay.

Background

Developing ELISA assays from critical reagents provides several attractive features over "one-time use" commercial kits. Purchasing the critical reagents in bulk is more cost effective, and it permits systematic development of a buffer-based assay which works well for exploratory investigations of a putative biomarker. In addition, by using bulk critical reagents, one can coat and block only the wells you need, thus conserving reagents. However, one disadvantage of a buffer-based assay is that any matrix interference from the test sample needs to be eliminated, as much as possible. This can often be an arduous and time-consuming process with no clear direction.

We started with a couple of standard assay buffers that we routinely try first with all new assays, in lieu of any recommendations from the literature. Typically we use one of the following: (1) 1% BSA (Bovine Serum Albumin), 0.05 % Tween-20 in PBS, pH 7.4; (2) 5% animal serum, 0.05% Tween-20, in PBS pH 7.4; and/or (3) a commercially available blocking buffer, with 0.05% Tween-20 in PBS. The initial observations with all three of these buffers were that plasma samples, assayed at multiple dilution factors, all produced raw OD (optical density) values well below that of blank buffer. From the literature, we were expecting concentrations of M-CSF to be in the range of about 25 – 200 pg/mL. With the initial LLOQ for the method set at 30 pg/mL, we suspected there was an interferent causing signal suppression and poor recovery of M-CSF in plasma.

Methods

Challenge #1: Percent Recovery of M-CSF in K2EDTA Plasma

In a stepwise fashion, normal mouse serum (NMS), normal equine serum (NES), and sodium chloride were added to the base buffer (commercially available SuperBlock Buffer in PBS, Thermo Scientific with 0.1% Tween-20) and IgM reducing assay diluent (ImmunoChemistry Technologies, LLC.) was added to each well of the microplate. Spike/recovery experiments in three individual lots of K_2EDTA plasma were performed to determine which buffer provided optimal recovery of M-CSF. A high salt wash buffer was used with a prolonged plate soaking step to improve replicate imprecision across the plate.

Assay Buffer	Plasma Lot		
	1	2	3
SuperBlock Buffer (PBS) with Tween-20 (Assay Buffer A)	25.8	35.2	32.6
Assay Buffer A + 2.5% Normal Mouse Serum (Assay Buffer B)	49.2	54.2	31.2
Assay Buffer B + 2.5% Normal Equine Serum (Assay Buffer C)	65.0	67.5	33.6
Assay Buffer C + 137mM NaCl (Assay Buffer D)	50.9	62.3	45.6
Assay Buffer D + Protein Based Assay Diluent	106.0	107.2	79.4

Figure 1: % Recovery of M-CSF in K2EDTA Plasma with various assay buffers.

Methods (Cont.)

Assay Buffer D increased the % recovery of M-CSF in plasma by as much as four times with a four-fold minimum required dilution (MRD). The MRD was previously eight-fold using just assay buffer C. With assay buffer C and addition of the protein-based assay diluent to each well, the LLOQ of the method was reduced from 30 pg/mL to 15.0 pg/mL. Therefore, use of this assay buffer/diluent combination resulted in a two-fold decrease in the LLOQ, while reducing the minimum required dilution in half, allowing us to measure concentrations of M-CSF four-times lower than previously possible.

Challenge #2: Replicate Imprecision

In ELISA assays, samples are often analyzed in duplicate, and replicate imprecision refers to the "closeness" of the two instrument response values, which are averaged to provide one reportable result. We typically have an acceptance criterion of ≤ 15.0% for replicate measurements. As can be seen in Figure 2, there are many samples which previously would have failed this criterion. Importantly, all but one of these samples were plasma samples, leading us to think that the protein content in the wells was leading to this issue.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.804	1.308	0.031	0.041	0.089	0.103	1.642	1.637	0.041	0.035	0.120	0.109
B	0.589	0.671	0.113	0.149	0.600	0.672	0.802	0.750	0.185	0.186	0.689	0.828
C	0.269	0.289	0.095	0.093	0.300	0.293	0.358	0.332	0.136	0.107	0.314	0.307
D	0.128	0.149	0.115	0.039	0.139	0.132	0.154	0.161	0.192	0.040	0.156	0.141
E	0.072	0.077	0.141	0.152	0.021	0.021	0.085	0.085	0.201	0.179	0.025	0.023
F	0.045	0.050	0.105	0.103	0.022	0.021	0.051	0.054	0.133	0.130	0.022	0.023
G	0.034	0.036	0.073	0.048	0.022	0.022	0.039	0.038	0.125	0.074	0.025	0.025
H	0.028	0.026	0.056	0.046	0.020	0.021	0.032	0.029	0.183	0.104	0.022	0.022

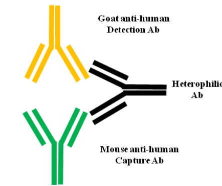
Figure 2: Plate map showing replicate imprecision in assay.

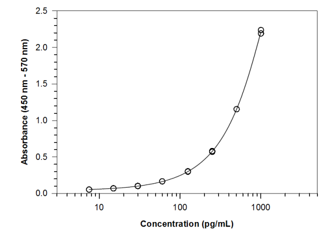
Matrix proteins are often quite sticky and the replicate imprecision issues can be due to poor washing of the microplate between steps. Matrix interferences in these assays are often times low affinity binders, with electrostatic interactions. Therefore, we increased the salt content of our wash buffer and added a prolonged soak step between the sample and detection antibody incubation, and all of the replicate imprecision issues were resolved. Replicate imprecision values are now typically <10.0% for all sample results with the modified plate washing procedure.

Method Characterization Results

Statistics	Nominal Standard Concentrations (pg/mL)						
	1000	500	250	125	60	30	15
Mean	1,004	504	247	123	61.1	30.2	15.7
n	6	6	6	6	6	6	5
SD	9.490	5.743	1.933	1.347	2.337	0.526	1.291
CV	0.9%	1.1%	0.8%	1.1%	3.8%	1.7%	8.2%
%Bias	0.4%	0.7%	-1.2%	-1.3%	1.8%	0.5%	4.9%

Figure 3: Calibration Curve Statistics





Characteristics	Statistics	Nominal Concentrations (pg/mL)				
		1000	750	150	45	15
		ULOQ	High	Medium	Low	LLOQ
# Results		9	9	9	9	9
# Runs		3	3	3	3	3
Accuracy	Mean	974	719	142	44.0	14.1
	% Bias	-2.6	-4.1	-5.4	-2.2	-6.0
Precision	Intra-Assay (%CV)	1.5	1.6	8.0	2.5	11.7
	Inter-Assay (%CV)	3.3	6.3	11.1	7.4	15.6
Total Error	%CV+%Bias	5.9	10.4	16.5	9.6	21.6

Figure 4: Precision and Accuracy

Sample #	Sample Description	RF (IU/mL)	Endogenous M-CSF pg/mL	Added M-CSF pg/mL	Plasma + M-CSF	% Recovery
1	Plasma A	140.4	117.115			
	Plasma A + M-CSF			670.184	896.519	116.3
2	Plasma B	465.5	126.892			
	Plasma B + M-CSF			670.184	826.197	104.3
3	Plasma C	588.3	31.460			
	Plasma C + M-CSF			670.184	727.015	103.8
4	Plasma D	230.7	19.896			
	Plasma D + M-CSF			670.184	688.352	99.7
5	Plasma E	>650	77.439			
	Plasma E + M-CSF			670.184	773.060	103.8
6	Plasma F	60.3	13.958			
	Plasma F + M-CSF			670.184	638.874	93.2
7	Plasma G	149.1	29.498			
	Plasma G + M-CSF			670.184	666.782	95.1
8	Plasma H	577.6	74.278			
	Plasma H + M-CSF			670.184	737.681	99.0

Figure 5: Spike/Recovery of M-CSF in Rheumatoid Arthritis Patient Plasma. RF = rheumatoid factor.

Conclusion

A method for the quantitation of M-CSF in K_2EDTA plasma using bulk critical reagents has been optimized to remove matrix interferences and improve overall replicate precision. Linearity, accuracy and precision, spike/recovery (selectivity), and stability (4 hours at RT) all met acceptance criteria. This systematic approach to remove matrix interferences can be used for similar assays.