#M1030

Process Parameters Affecting Reproducibility and Reliability When Calibrating Immunoassays to International Reference Standards

Christopher Campbell, Christopher Shelburne, Joseph Manimala, Amandeep Kaur, Qian Ning, Prachi Gupta, Ed Massuda, David Stewart, Pankaj Oberoi, and Jacob N. Wohlstadter

Meso Scale Discovery, Rockville, Maryland, USA

1 Abstract

Purpose: Many commercial immunoassays are available for quantifying biomarkers. Comparing data across platforms requires accurate calibration against industry-accepted standards. Although NIBSC/WHO reference standards (http://www.nibsc.org/) are available for many immunoassays, commonly accepted methods have not been established for designing and executing reliable calibration protocols. Data collected using too few runs, days, or operators may lead to inaccurate calibration; however, another significant factor is the stability of the reference standards when stored according to instructions provided in the package insert. Here we demonstrate a multi-day and multi-operator calibration protocol designed to obtain reliable calibration to international reference standards.

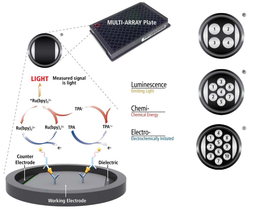
Methods: We characterized sources of variability affecting calibration of Proinflammatory Panel 1 (human) V-PLEX® cytokine standards against NIBSC/WHO reference standards. Tested assays included IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13 and TNFα. Specifically, we assessed the variability of assay performance and reference standards by comparing results from multiple runs, days, operators, and vials. In addition, we evaluated storage stability (2–8°C and ≤-70°C) of reconstituted reference standards.

Results: Compared to using aliquots that were freshly reconstituted from lyophilized reference standards, calibration using frozen aliquots created the largest variability. NIBSC/WHO product inserts generally do not advise against freezing reconstituted material and in some cases recommend storing frozen aliquots at <-40°C. However, after a single freeze–thaw cycle, recovery relative to freshly prepared aliquots was <80% for most standards. In contrast, after a week of refrigeration (2–8°C), recovery was 80–120% for most standards and 65–135% for all standards. Vial-to-vial and operator-to-operator variability was low for all reference standards. We show that accurate calibration can be achieved when the protocol involves evaluation of international reference standards using freshly reconstituted lyophilized standards or reconstituted standards that have been demonstrated to be stable when stored at 2–8°C to allow multiple runs over multiple days.

Conclusions: The process of calibrating immunoassays to international reference standards is impacted by numerous parameters affecting reproducibility and reliability. When designing calibration protocols and evaluating the reliability of calibration factors, it is important to consider the storage stability of reconstituted reference material as well as data collection parameters.

2 Methods

Lyophilized NIBSC/WHO international standards were reconstituted and aliquots were prepared according to instructions provided by the vendor. Standards were diluted to a working concentration near the upper limit of the quantifiable range of Proinflammatory Panel 1 (human) V-PLEX assays then serially diluted 4-fold to obtain a total of 4 dilutions per standard. Proinflammatory Panel 1 (human) calibrators were reconstituted and aliquots were prepared according to the Proinflammatory Panel 1 (human) protocol. Stimulated samples with elevated levels of native analytes were prepared from whole blood or peripheral blood lymphocytes challenged with LPS. Samples were frozen until used. All assays were evaluated with the Proinflammatory Panel 1 (human) Kit using MSD® ECL technology and the same three step protocol:



Electrochemiluminescence Technology

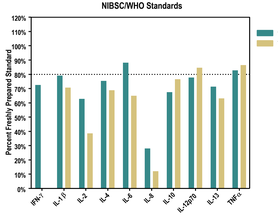
- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- Carbon electrode surface has 10X greater binding capacity than polystyrene wells.
- Surface coatings can be customized.

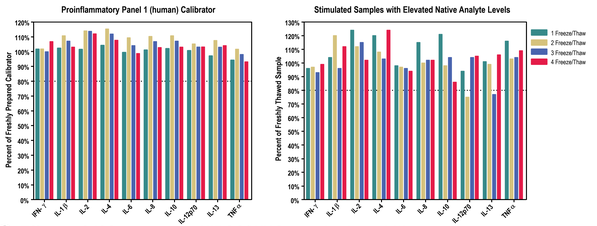
Protocol

1. Add calibrator, control or sample (25 µL/well). Incubate 2 hours at room temperature (RT).
2. Wash and add detection antibody solution (25 µL/well). Incubate 1 hour at RT.
3. Wash and add read buffer (150 µL/well). Analyze with MSD instrument.

3 Effects of freeze-thaw on NIBSC/WHO standards stability

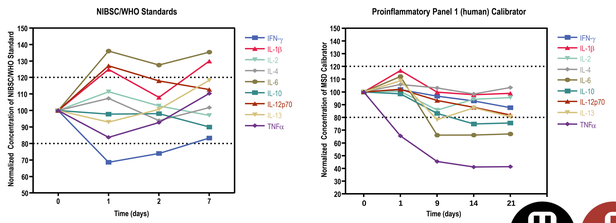
The effects of freeze/thaw on the stability of NIBSC/WHO standards, Proinflammatory Panel 1 (human) calibrators, and native analytes in stimulated samples was determined. Data from NIBSC/WHO standards and Proinflammatory Panel 1 (human) calibrators is expressed as percent of freshly prepared samples. Data from stimulated samples is expressed as percent of freshly thawed samples. As shown, NIBSC/WHO standards demonstrate a significant loss of activity relative to MSD internal calibrators or native samples after one freeze/thaw.





4 Effects of 4°C storage on NIBSC/WHO standards stability

The effects of storing NIBSC/WHO and Proinflammatory Panel 1 (human) calibrators at 4°C over time were evaluated in this study. Data is expressed as percent of results obtained with freshly prepared standards. After 24 hours, NIBSC/WHO standards (especially IL-6 and IFN-γ) demonstrated variability (maximal change up to 36%) that appeared to stabilize after 1 day. Afterwards, each standard was stable for up to 1 week. Most Proinflammatory Panel 1 (human) calibrators were stable over time with the exception of TNFα and IL-6.



5 Run variability of NIBSC/WHO standards

Run variability of freshly prepared NIBSC/WHO standards and Proinflammatory Panel 1 (human) calibrators were evaluated. Three individual operators analyzed NIBSC/WHO standards in the same day. Six individual operators analyzed Proinflammatory Panel 1 (human) calibrators in 28 runs over a three day period. Mean (pg/mL), intra-run %CV, inter-operator %CV and inter-run %CV are displayed for each analyte. In all cases, inter-operator %CV is <15%.

NIBSC/WHO Standards

Assay	Mean (pg/mL)	Intra-run %CV	Inter-operator %CV	Inter-run %CV (n=4)
IFN-γ	822.4	5.1	9.6	20.5
	718.8	3.1		
	686.1	1.7		
IL-1β	60.6	4.4	5.1	18.9
	64.9	6.3		
	62.8	3.3		
IL-4	6.31	8.8	12.7	5.2
	13.6	4.1		
	16.5	6.3		
IL-6	84.9	11.2	14.2	10.9
	64.0	7.7		
	82.7	14.8		
IL-10	46.9	4.2	11.9	3.2
	36.4	3.5		
	42.4	3.2		
IL-12p70	46.6	5.1	13.1	16.9
	35.2	2.8		
	41.3	2.6		
IL-13	125.1	25.6	14.1	10.3
	96.0	18.8		
	127.4	21.9		
TNFα	37.0	2.8	10.8	14.6
	29.8	0.5		
	34.0	2.3		

Proinflammatory Panel 1 (human) Calibrators

Assay	Mean (pg/mL)	Intra-run %CV	Inter-operator %CV	Inter-run %CV (n=3)
IFN-γ	330.2	6.3	12.8	3.9
	274.6	4.8		
	257.4	6.5		
	240.7	11.2		
	267.9	4.1		
	265.4	16.3		
IL-1β	131.4	5.3	6.7	4.7
	124.6	6.2		
	116.2	5.3		
	118.0	3.7		
	123.3	2.8		
	129.5	9.6		
IL-4	57.7	8.1	10.3	2.9
	52.1	9.2		
	50.9	13.7		
	41.3	13.9		
	47.9	12.3		
	46.8	28.0		
IL-6	212.1	3.6	5.5	8.1
	219.9	8.5		
	191.1	3.5		
	204.8	3.5		
	211.7	5.2		
	217.2	9.4		
IL-10	86.6	5.8	8.4	5.2
	78.3	6.6		
	70.1	5.8		
	69.6	7.1		
	78.5	8.6		
	73.0	14.7		
IL-12p70	111.0	9.2	9.6	5.5
	98.5	5.0		
	90.0	3.5		
	84.3	11.0		
	95.0	5.8		
	91.0	21.3		
IL-13	137.4	3.7	4.1	6.5
	142.8	4.7		
	131.9	3.0		
	129.4	4.8		
	136.4	2.1		
	145.5	8.1		
TNFα	88.3	9.2	9.6	6.9
	76.6	5.6		
	67.8	3.0		
	69.3	4.2		
	71.9	2.7		
	69.6	13.5		
IL-2	377.4	2.8	7.0	8.0
	396.5	4.9		
	334.9	4.8		
	330.9	5.0		
	370.2	3.4		
	370.3	13.8		
IL-8	145.1	7.0	5.6	5.7
	136.3	3.1		
	122.3	5.3		
	126.9	3.5		
	132.8	2.7		
	132.0	9.9		

6 Vial to vial variability of NIBSC/WHO standards

Vial to vial variability of freshly prepared NIBSC/WHO standards was evaluated by reconstituting and analyzing 2 vials of each standard by one operator on the same day. Vial to vial variability of freshly prepared Proinflammatory Panel 1 (human) calibrators was evaluated by reconstituting and analyzing 22 vials of each standard by one operator on the same day. Inter-vial %CVs are displayed for each analyte. Except for IFN-γ, all analytes showed consistent vial to vial reproducibility. The reason for the instability of the NIBSC/WHO IFN-γ standard, which is partially purified from supernatants of lectin activated human leukocytes, remains unclear. Alternate NIBSC/WHO recombinant IFN-γ standards will be tested in the future.

Assay	NIBSC/WHO Standards (n=2) Inter-Vial %CV	Proinflammatory Panel 1 (human) Calibrator (n= 22) Inter-Vial %CV
IFN-γ	49.3	3.7
IL-1β	1.8	4.6
IL-4	8.6	5.7
IL-6	3.9	5.9
IL-10	3.3	4.3
IL-12p70	0.2	5.3
IL-13	3.2	4.1
TNFα	8.6	4.2

7 Calibration of MSD Assays to NIBSC/WHO standards

Based on our new reference methodology for handling NIBSC/WHO standards, we performed a new calibration of our MSD calibrators with NIBSC/WHO standards. Results are based on multi-run averages using freshly prepared and refrigerated aliquots of NIBSC/WHO standards from multiple vials. Concentration ratios were calculated by dividing the measured concentration of an MSD calibrator by the concentration of the associated NIBSC/WHO reference (if available) reported in the NIBSC/WHO standards datasheet. Since NIBSC/WHO standards are reported in International Units (IU), concentration ratios were transformed into a conversion factor (pg/IU) based on NIBSC/WHO assigned analyte activity (pg/IU) (obtained from datasheet). Therefore, to anchor MSD calibrators to NIBSC/WHO IU standards, the concentration of an MSD calibrator should be divided by the conversion factor to achieve NIBSC/WHO standard values.

Assay	NIBSC/WHO Catalog Number	Ratio	Conversion Factor (pg/IU)	Runs (n)	Inter-run %CV
IFN-γ	82/587	ND	770	11	22.63
IL-1β	86/680	0.47	4.68	8	11.73
IL-2	86/504	1.03	78.2	7	4.63
IL-4	88/656	0.32	32.3	8	7.95
IL-6	89/548	0.43	4.32	8	11.86
IL-8	89/520	0.84	840	3	8.18
IL-10	93/772	0.53	106	8	7.74
IL-12p70	95/544	0.42	42.2	8	10.38
IL-13	94/622	0.34	344	8	10.63
TNFα	88/786	0.46	9.79	8	8.97

8 Conclusion

Proinflammatory Panel 1 (human) V-PLEX assays have undergone rigorous validation, including running NIBSC/WHO reference standards that are typically used to normalize results among different labs. Our development of Proinflammatory Panel 1 (human) assays identified challenges in handling these reference standards. Improper handling could lead to inaccurate calibration and lead to lab-to-lab variability. We found that while most Proinflammatory Panel 1 (human) calibrators and samples are stable when subjected to freeze-thaw cycles, the NIBSC/WHO standards could degrade by as much as 70% when subjected to only one freeze-thaw cycle. The NIBSC/WHO standards were generally consistent vial to vial and stable when stored at 4°C for up to a week. We have established thorough reagent handling procedures along with a multi-day analysis of the NIBSC/WHO standards to assign a conversion ratio between the Proinflammatory Panel 1 (human) V-PLEX calibrators and the NIBSC/WHO standards. These handling procedures improve reproducibility and reliability when calibrating Proinflammatory Panel 1 (human) V-PLEX assays to International Reference Standards.

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