Verification of the Hamilton Microlab® STARlet for Use with the SMCxPRO™ and Erenna® Immunoassay Systems Powered by Single Molecule Counting (SMC™) Technology

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Introduction

Cytokines are signaling molecules that mediate and regulate the immune system. Interleukins, which regulate immune and inflammatory responses, and interferons, which are responsible for adaptive immunity, are two broad cytokine categories that are particularly challenging to measure using traditional ELISAs or ligand binding assays because they are present in low levels within the human body.

An alternative to quantifying these low-level cytokines is the patented Single Molecule Counting (SMC™) Technology from MilliporeSigma. The SMC™ Technology method is similar to a traditional ELISA and offers reduced background signal and increased detection signal in comparison. In process, target analytes in solution are captured onto an antibody-bound plate or microbead. Fluorescently labeled detection antibodies are then added to the immune complex to translate each biomarker into a signal. During a modified elution step, the bound antibodies are released from the immune complex where the fluorescent signal from single, tagged molecules are detected by either the SMCxPRO™ or Erenna® instruments. The total signal detected is calculated as a direct indication of biomarker levels, with a limit of detection down to < 1 fM*.

When manually performed, the SMC™ immunoassays are labor-intensive. This prevents researchers from focusing on high-value activities such as result interpretation, thus reducing overall laboratory efficiency. Manual methods also introduce risk of operator error and elements of variability from one user to the next and among subsequent assay runs.

Here, we demonstrate use of the Erenna® Immunoassay System with SMC™ Immunoassays integrated onto the Hamilton Microlab® STARlet liquid handling workstation in order to create a hands-free, automated, assay-ready workflow (see Workflow at end). The STARlet is equipped with up to four independent air displacement pipetting channels for high precision and reliability without reagent crossover. Compressed O-Ring Expansion (CO-RE®) Technology creates an air-tight seal between the disposable tips and pipetting channel mandrels without using mechanical force, to maximize sample care and integrity, and also ensure accurate, reproducible liquid level dispensing. Barcode reading provides full sample tracking and eliminates the risk of sample mishandling or manual

Benefits-Based Highlights

- Robust, sensitive, and reproducible method to quantify biomarker concentration without sources of error and variability associated with manual processing.
- Refocus manual labor on high-value activities such as result interpretation.

**Determined from calibrator set: 0–300 fM of 150 kD antibody labeled with fluorophore.*

documentation errors. Two ELx405™ HT Microplate Washers (BioTek Instruments, Winooski, VT) and four heated shakers were integrated into the deck of the STARlet for added workflow efficiency and convenience (see Deck Layout at end). Finally, in order to facilitate user-friendly operation, minimize operator intervention, and reduce input errors, the STARlet software was pre-programmed with the SMC™ workflow steps to create a standardized solution.

Using plasma samples and controls, we demonstrate that the assay-ready automated workstation delivers results on par with those achieved through manual methods, while maximizing assay reproducibility, reducing active labor time, and eliminating risks of error and variability from manual intervention.

Materials and Methods

Automated and manual workflows were compared using the SMC™ Interleukin 6 (IL-6) Immunoassay Kit (P/N 30-0572-01-TED), SMC™ Tumor Necrosis Factor (TNF-α) Immunoassay Kit (P/N 30-0571-01-TED), and SMC™ Interleukin 1-β (IL-1β) Immunoassay Kit (P/N 30-0573-01-TED) from Singulex. In each assay, the manufacturer's protocol was followed[1–3].

Standard, Sample, and Control Preparation

Standard protein curves were created manually as follows. IL-6 standard protein was thawed and diluted to 100 pg/mL in standard diluent to make the top standard, followed by ten 2-fold serial dilutions, down to 0.1 pg/mL. TNF-α standard protein was thawed and diluted to 200 pg/mL in standard diluent to make the top standard, followed by ten 2-fold serial dilutions, down to 0.31 pg/mL. IL-1β standard protein was diluted to 50 pg/mL in standard diluent to make the top standard, followed by ten 2-fold serial dilutions, down to 0.05 pg/mL. Each standard curve also included a zero blank.

Human K2 EDTA plasma samples from five healthy individuals (BioreclamationIVT P/N HMPLEDTA2, Westbury, NY) and 3 plasma controls (MilliporeSigma, Hayward, CA) were tested as described in Table 1. Three vials of each sample and plasma control were thawed, lightly mixed, and filtered through a 96-well, 1.2 µm Durapore® membrane filter plate (Millipore, #MSBVN1210) according to each kit protocol.

SMC™ Immunoassay Workflow

For each automated assay type, a 4 Row, Pyramid Bottom 292 mL High Profile Reagent Reservoir (E&K Scientific, P/N EK-2216) was loaded onto the STARlet with assay-specific reagents. Using 300 µL conductive non-filtered CO-RE tips (P/N 235950), a total of 100 µL of microparticles per well were added to four 96-well v-bottom polypropylene microplates (E&K Scientific, P/N EK2470, Santa Clara, CA), followed by 100 µL of each respective 12-point standard protein curve in triplicate. For each assay kit, 100 µL of sample or plasma control filtrate was added to each of the four microplates. The microplates were then incubated on the STARlet deck for two hours at 25ºC with shaking to allow binding of the target biomarker. The assay plates were then transferred to the microplate washer, where the microbeads were magnetically retained, and unbound material was removed in a single wash step. After washing, 20 µL Alexa Fluor 647-labeled detection reagent was added to the wells, using 50 µL conductive non-filtered CO-RE tips (P/N 235947), and the microplates were incubated for one hour in order to bind the microbead-captured analyte. After incubation, the assay plates were again transferred to the microplate washer, where the microbeads were magnetically retained and washed four times in order to remove any unbound detection reagent. The microparticles were then automatically transferred from the 96-well assay microplates to new microplates to avoid eluting non-specific plate bound detection reagent. Detection reagent specifically bound to the target analyte was then eluted and transferred to a 384-well polypropylene microplate (ThermoFisher Scientific P/N 264573, Waltham, MA). The 384-well microplate was manually transferred to the Erenna® Instrument for detection. Alternatively the plate could also be read on the SMCxPRO™ Instrument.

The entire workflow was also performed using manual methods and one microplate per assay.

Three signal outputs were obtained from the Erenna® Instrument: Detected Events (DEs; low end signal), Event Photons (EPs; low end and mid-range signal), and Total Photons (TPs; high end signal). Using the SgxLink™ algorithm, unknown concentrations were interpolated from the standard curve.

1. SMC™ Human IL-6 High Sensitivity Immunoassay Kit: Immunoassay kit for the quantitative determination of Interleukin 6 (IL-6) in human EDTA plasma. Singulex: Alameda, CA. Dec 14, 2017. Kit P/N: 30-0572-01-TED.

2. SMC™ Human TNF-α Immunoassay kit for the quantitative determination of Tumor Necrosis Factor (TNFα) in human EDTA plasma. Singulex: Alameda, CA. May 15, 2017. Kit P/N: 30-0571-01-TED.

3. SMC™ IL-1β High Sensitivity Immunoassay Kit: Immunoassay kit for the quantitative determination of Interleukin 1β (IL-1β) in human EDTA plasma. Singulex: Alameda, CA. Dec 14, 2017. Kit P/N: 30-0573-01-TED.

Results and Discussion

Using data obtained from the single, manually processed microplate, and the four replicate microplates that were automatically processed on the STARlet liquid handling system, an interpolated average for each protein standard concentration was calculated and plotted as a standard curve. Interpolated averages from each sample and control that were manually and automatically processed were then plotted against the standard curve. When comparing manual and automated results, analyte concentration, SD, and CV values produced robotically are all on par with those produced manually. All samples tested in the IL-6 and TNF-α assays resulted in interpolated values well above the sensitivity of the assays. IL-6 and TNF-α protein concentrations are generally more abundant in human samples, and assay results are well above the lower limit of quantitation (LLOQ) of the SMC™ assays. But IL-β protein concentrations in human samples are known to be very low, resulting in IL-β assay results near the LLOQ of the assay (0.2 pg/mL) with some below. However, samples that are quantifiable did show a good correlation between the Hamilton and manual method assay methods.

IL-6 Results

The IL-6 slope approaches 1, indicating a strong correlation of interpolated IL-6 values between manual and automated runs of both plasma and control samples. Each of the four plates produced by automated means had similar interpolated IL-6 values for both plasma samples and controls.

LLOQ = 0.08 pg/mL; see Table 2 and Figure 1 for data.

TNF-α Results

The TNF-α slope approaches 1, indicating a strong correlation of interpolated TNF-α values between manual and automated runs of both plasma and control samples. Each of the four plates produced by automated means had similar interpolated TNF-α values for both plasma samples and controls.

LLOQ = 0.2 pg/mL, LOD 0.05 pg/mL; see Table 3 and Figure 2 for data.

IL- β Results

The IL-1β slope approaches 1, indicating a strong correlation of interpolated IL-1β values between manual and automated runs of both plasma and control samples. Samples in which the signal was below the LLOQ are listed as "not quantifiable."

LLOQ = 0.2 pg/mL, LOD 0.1 pg/mL; see Table 4 and Figure 3 for data.

Conclusion

The SMCxPRO™ and Erenna® Systems with SMC™ Technology allow researchers to detect and monitor changes in extremely low levels of established disease biomarkers such as interleukins and interferons. When the assay technology is integrated as an automated workflow using the Microlab® STARlet, results are comparable to those obtained using manual methods, and within acceptable limits as established by the manufacturer. The automated workflow eliminates the risk of errors and variability due to manual manipulations, and also allows researchers to refocus their efforts on high-value activities.

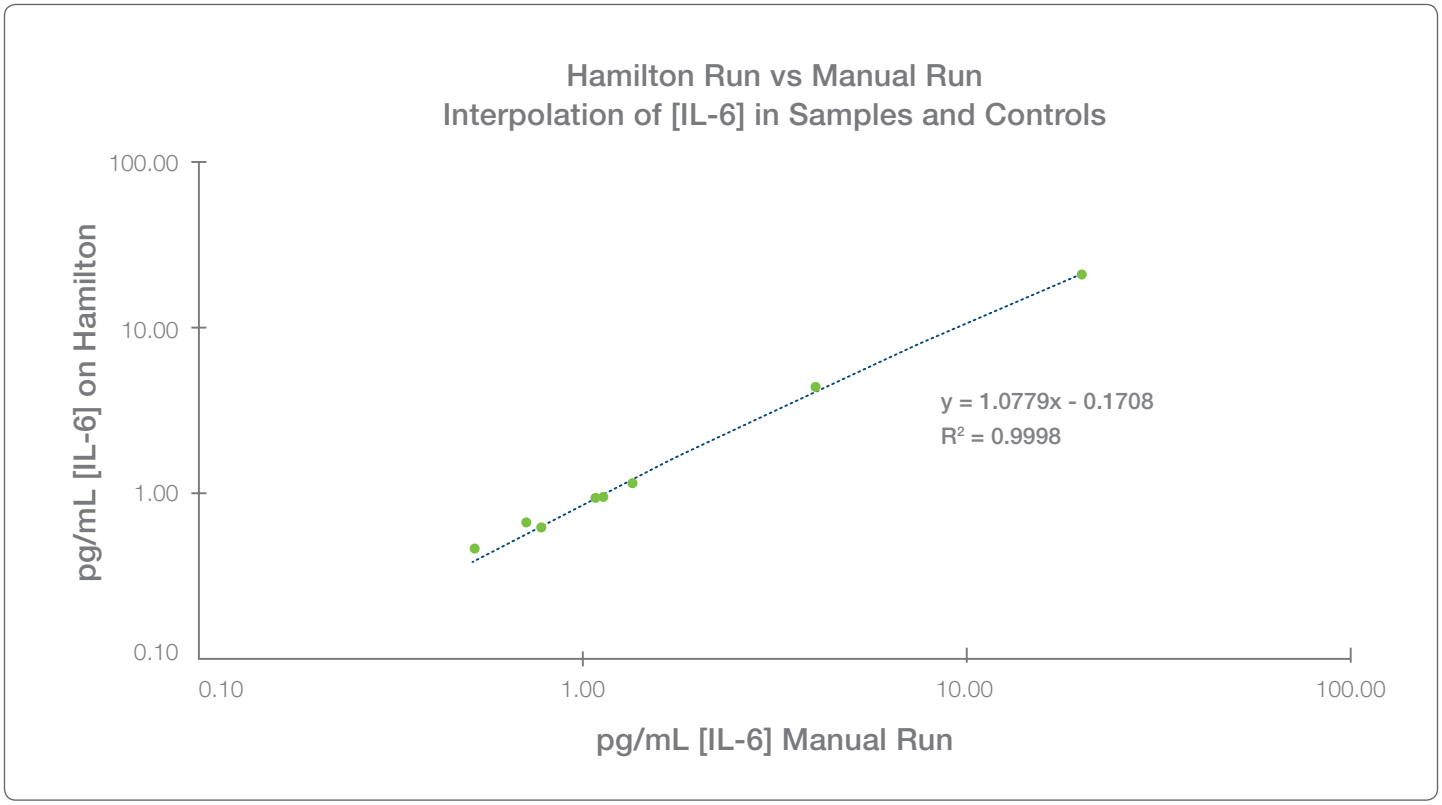


Figure 1: Correlation of interpolated Interleukin-6 assay values between manual and automated methods.

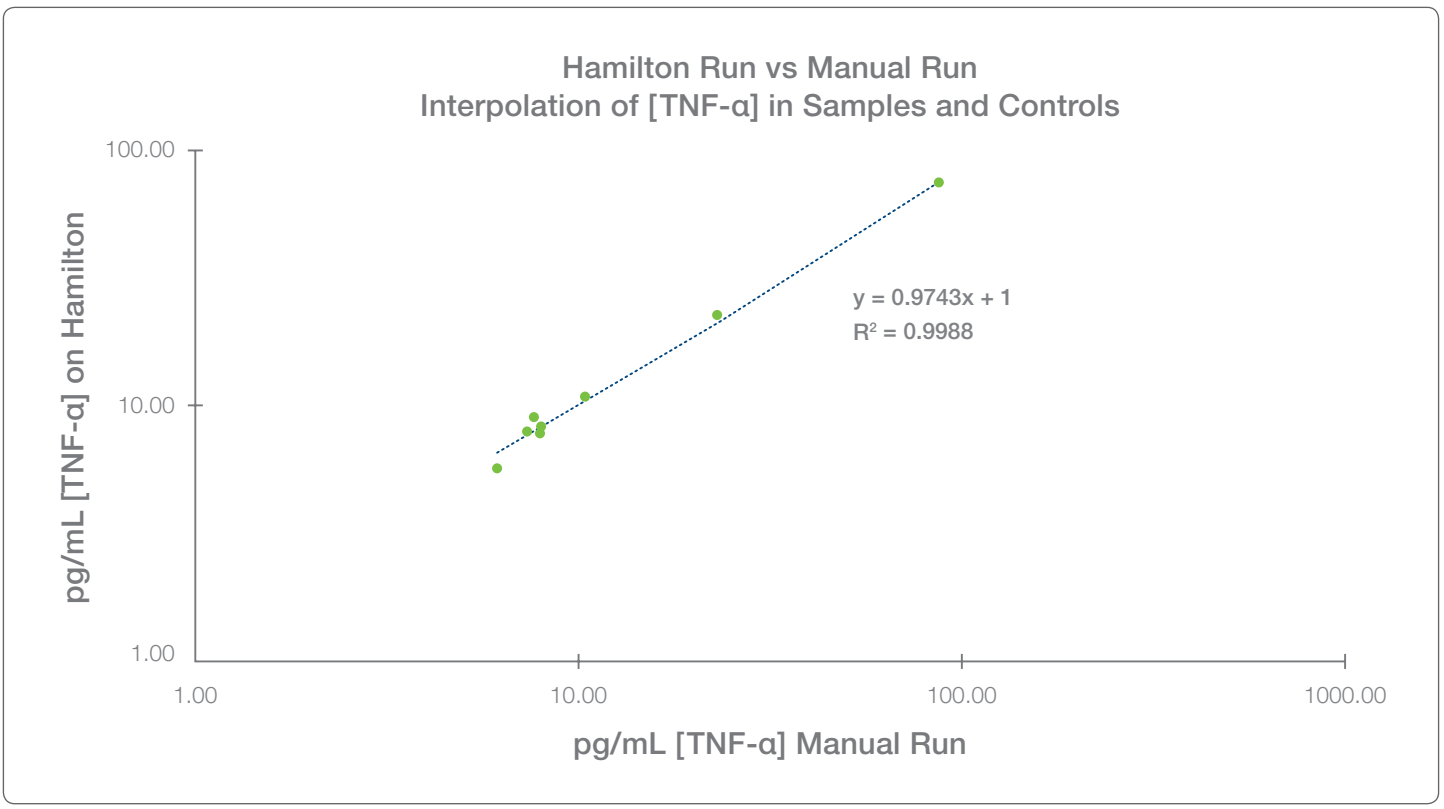


Figure 2: Correlation of interpolated Tumor Necrosis Factor-α assay values between manual and automated methods.

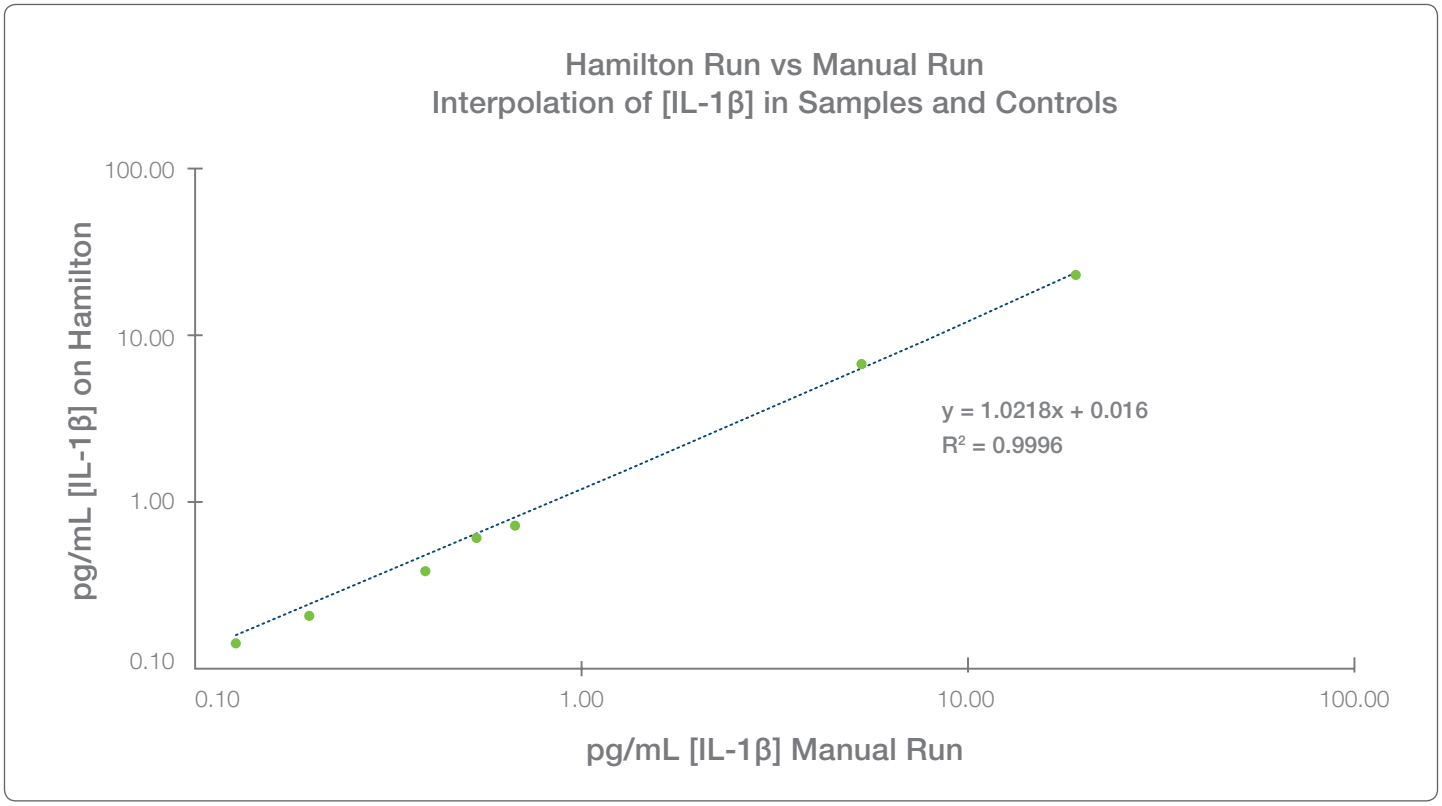


Figure 3: Correlation of interpolated Interleukin-1β assay values between manual and automated methods.

Table 1: Plasma Test and Control Sample Information

Assay Type	K2 EDTA Human Plasma Samples Lot #	Control 1 MilliporeSigma Catalog #, Lot #	Control 2 MilliporeSigma Catalog #, Lot #	Control 3 MilliporeSigma Catalog #, Lot #
IL-6	BRH884229	Custom, Lot 1410206	Custom, Lot 1410207	Custom, Lot 1410208
IL-6	BRH1152533	Custom, Lot 1410206	Custom, Lot 1410207	Custom, Lot 1410208
IL-6	BRH1152534	Custom, Lot 1410206	Custom, Lot 1410207	Custom, Lot 1410208
IL-6	BRH1152535	Custom, Lot 1410206	Custom, Lot 1410207	Custom, Lot 1410208
IL-6	BRH1152536	Custom, Lot 1410206	Custom, Lot 1410207	Custom, Lot 1410208
TNF-α	BRH1457831	Custom, Lot 1410209	Custom, Lot 1410210	Custom, Lot 1410211
TNF-α	BRH1457832	Custom, Lot 1410209	Custom, Lot 1410210	Custom, Lot 1410211
TNF-α	BRH1457833	Custom, Lot 1410209	Custom, Lot 1410210	Custom, Lot 1410211
TNF-α	BRH1457834	Custom, Lot 1410209	Custom, Lot 1410210	Custom, Lot 1410211
TNF-α	BRH1457839	Custom, Lot 1410209	Custom, Lot 1410210	Custom, Lot 1410211
IL-1β	BRH1152524	Custom, Lot 1003069	Custom, Lot 1003070	Custom, Lot 1003071
IL-1β	BRH1152525	Custom, Lot 1003069	Custom, Lot 1003070	Custom, Lot 1003071
IL-1β	BRH1152527	Custom, Lot 1003069	Custom, Lot 1003070	Custom, Lot 1003071
IL-1β	BRH1152529	Custom, Lot 1003069	Custom, Lot 1003070	Custom, Lot 1003071

Table 2: Comparison of Manual Versus Automated Processing of the Interleukin-6 Assay Using Plasma

		ID	Lot#	N	Interpolated IL-6 Avg pg/mL	SD	%CV
Manual Process	Samples	JA1	BRH884229	3	1.14	0.14	12
	Samples	JA2	BRH1152533	2	1.08	0.11	10
	Samples	JA3	BRH1152534	3	1.35	0.24	18
	Samples	JA4	BRH1152535	1	0.71	–	–
	Samples	JA5	BRH1152536	3	0.78	0.08	11
	Controls	C01	1410206	3	0.52	0.04	7
	Controls	C02	1410207	3	4.04	0.37	9
	Controls	C03	1410208	3	19.86	0.82	4
Automated Process	Samples	JA1	BRH884229	10	0.96	0.11	11
	Samples	JA2	BRH1152533	11	0.94	0.12	13
	Samples	JA3	BRH1152534	11	1.17	0.13	11
	Samples	JA4	BRH1152535	7	0.68	0.02	2
	Samples	JA5	BRH1152536	12	0.62	0.03	5
	Controls	C01	1410206	12	0.46	0.04	8
	Controls	C02	1410207	12	4.38	0.26	6
	Controls	C03	1410208	12	21.21	1.04	5

Inter-Method Precision			
	Avg	SD	%CV
	1.05	0.13	12
	1.01	0.10	10
	1.26	0.13	10
	0.7	0.03	4
	0.7	0.11	16
	0.49	0.05	9
	4.21	0.24	6
	20.53	0.95	5

Table 3: Comparison of Manual Versus Automated Processing of the Tumor Necrosis Factor-α Assay Using Plasma

		ID	Lot#	N	Interpolated TNF-α Avg pg/mL	SD	%CV
Manual Process	Samples	JA1	BRH1457831	3	5.81	0.10	2
	Samples	JA2	BRH1457832	3	7.44	0.00	0
	Samples	JA3	BRH1457833	3	9.73	0.40	4
	Samples	JA4	BRH1457834	3	7.50	0.20	3
	Samples	JA5	BRH1457839	3	6.90	0.20	3
	Controls	C01	1410209	3	7.23	0.10	1
	Controls	C02	1410210	3	20.91	0.70	3
	Controls	C03	1410211	3	76.08	0.50	1
Automated Process	Samples	JA1	BRH1457831	12	5.78	0.43	7
	Samples	JA2	BRH1457832	12	7.87	0.58	7
	Samples	JA3	BRH1457833	12	11.03	0.39	4
	Samples	JA4	BRH1457834	12	8.42	0.61	7
	Samples	JA5	BRH1457839	12	8.02	0.75	9
	Controls	C01	1410209	12	9.12	0.41	4
	Controls	C02	1410210	12	22.75	1.43	6
	Controls	C03	1410211	12	74.64	3.70	5

Inter-Method Precision			
	Avg	SD	%CV
	5.80	0.02	0
	7.65	0.31	4
	10.38	0.92	9
	7.96	0.65	8
	7.46	0.79	11
	8.17	1.34	16
	21.83	1.30	6
	75.36	1.02	1

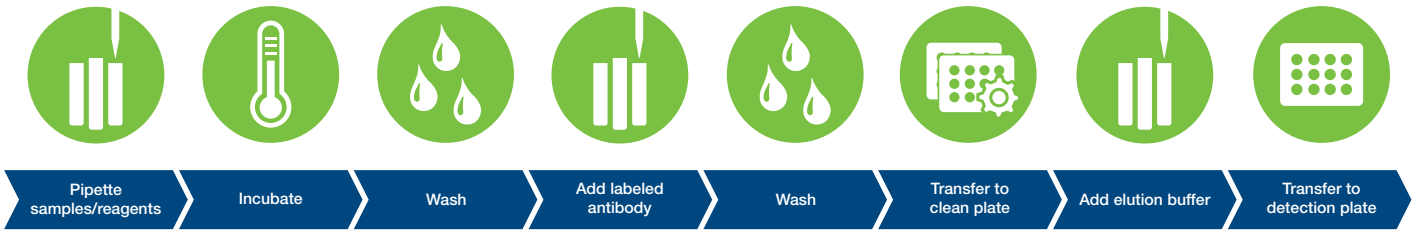
Table 4: Comparison of Manual Versus Automated Processing of the Interleukin-1β Assay Using Plasma

		ID	Lot#	N	Interpolated IL-1β Avg pg/mL	SD	%CV
Manual Process	Samples	JA1	BRH1152524	2	< LLOQ*	–	–
	Samples	JA2	BRH1152525	2	0.53	0.10	16
	Samples	JA3	BRH1152527	2	0.39	0.00	3
	Samples	JA4	BRH1152529	2	< LLOQ*	–	–
	Controls	C01	1003069	3	0.66	0.00	4
	Controls	C02	1003070	3	5.06	0.50	11
	Controls	C03	1003071	3	17.91	0.20	1
Automated Process	Samples	JA1	BRH1152524	10	< LLOQ*	–	–
	Samples	JA2	BRH1152525	11	0.53	0.05	9
	Samples	JA3	BRH1152527	10	0.34	0.06	18
	Samples	JA4	BRH1152529	9	< LLOQ*	–	–
	Controls	C01	1003069	12	0.63	0.04	6
	Controls	C02	1003070	11	5.48	0.36	7
	Controls	C03	1003071	12	18.24	0.32	2

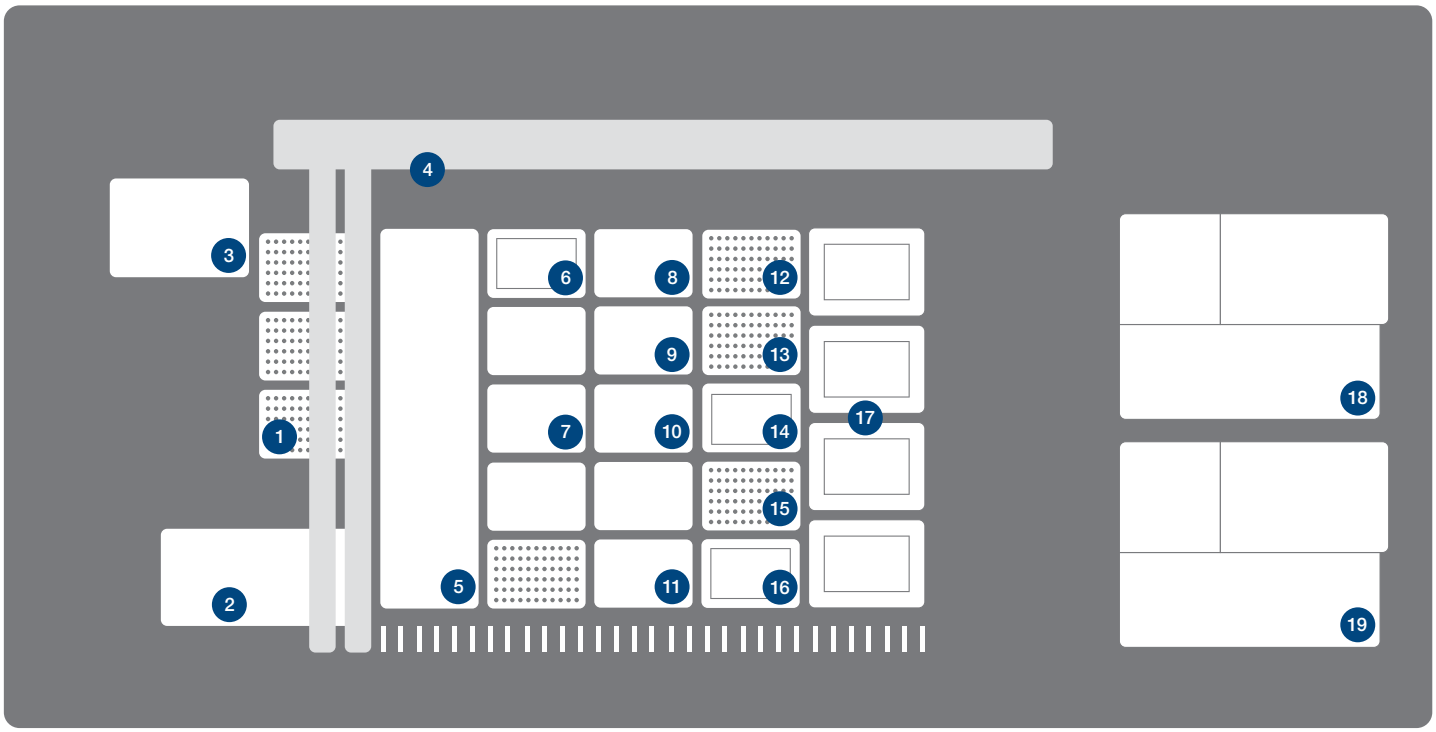
Inter-Method Precision	Avg	SD	%CV
	0.19	0.01	5
	0.53*	0.00	0
	0.37	0.04	10
	0.13	0.00	1
	0.65*	0.02	3
	5.27	0.30	6
	18.08	0.23	1

** < LLOQ — Interpolated sample valule is below the 0.20 pg/mL limit of quantification*

Automated Workflow for the SMCxPRO™ and Erenna® Immunoassay System on the Microlab® Starlet



Microlab® STARlet Deck Layout for the SMCxPRO™ / Erenna® Immunoassay



1. Nested Tip Rack Carrier
2. MPH Waste
3. 96 MPH
4. iSWAP
5. Nested Tip Rack Carrier
6. Lid Park Position
7. 96-Well Sample Plates
8. Lid Park Position
9. 384-Well Sample Plate
10. 96-Well Sample Plates
11. 96-Well Sample Plates
12. Lid Park Position
13. Reagent Trough—4 Rows
14. Magnetic Stand*
15. 96-Well Sample Plate
16. Magnetic Stand*
17. Thermo-shakers
18. Plate Washer*
19. Plate Washer*

** See ordering information below.*

Ordering Information

Product	P/N	Description	Vendor
Hamilton STARlet	0203901-000	STARlet with Integrated BioTek Washers	Hamilton
Magnetic Plates	90-0003-02	Magnetic Plate for STARlet and BioTek 405TS	MilliporeSigma
BioTek Plate Washer	405TSUVS	BioTek 405 Select Microplate Washer	BioTek Inc.

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