APPLICATION NOTE

Multiplex protein analysis using LUNARIS Kits and the CYTENA C.WASH plate washer and liquid dispenser

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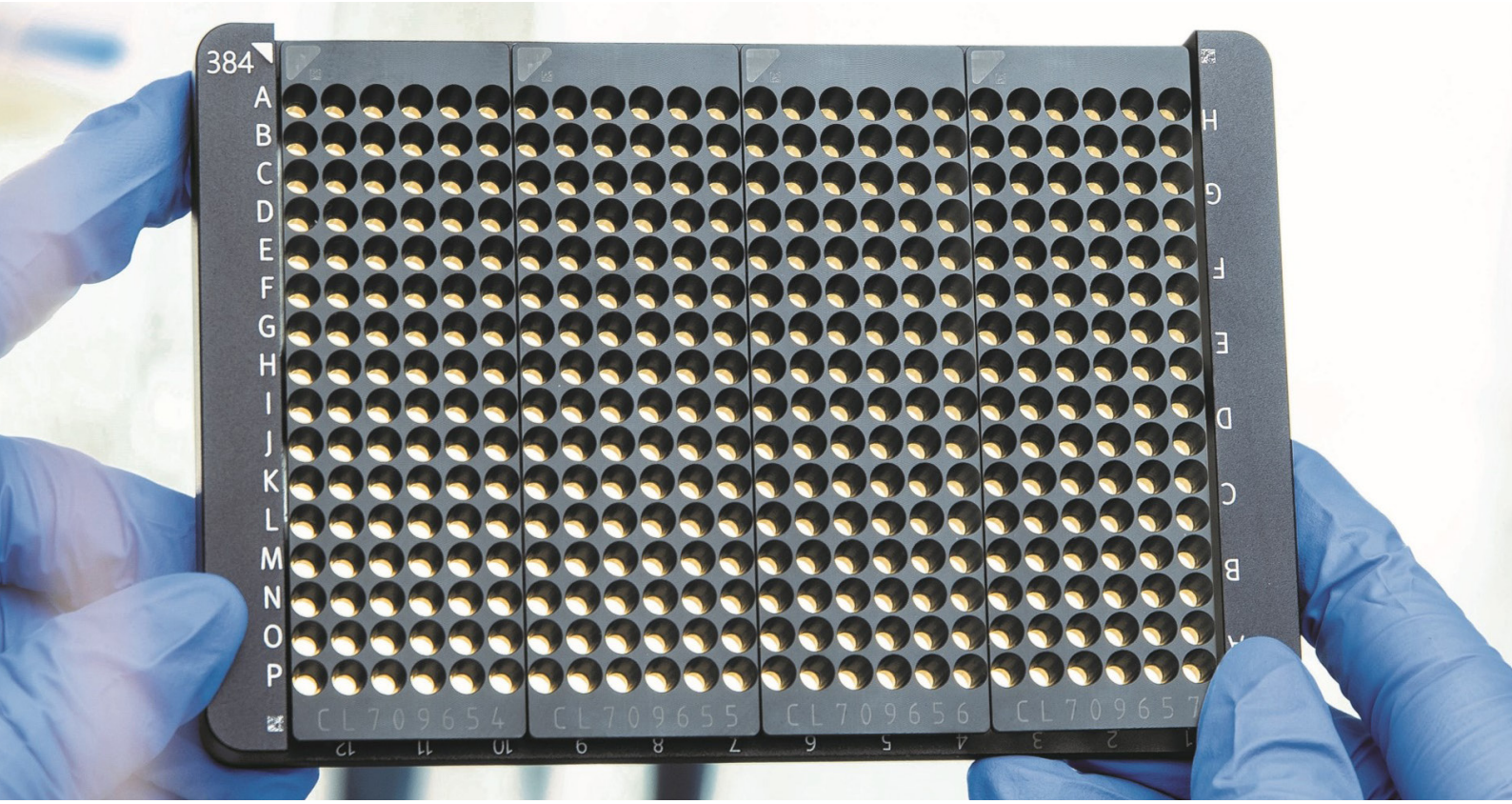
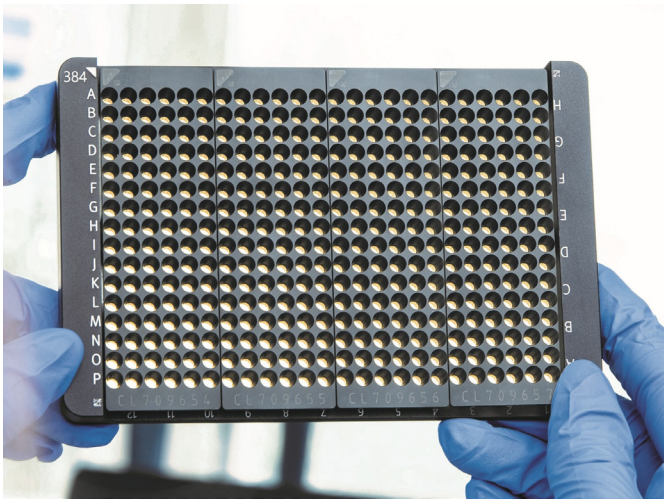


A semi-automated workflow for processing LUNARIS multiplex assays in life science research and routine lab procedures

Abstract

To ensure uniform assay performance and guarantee the highest sensitivity, an efficient and reproducible washing procedure is crucial for immunoassays. Therefore, LUNARIS™ multiplex assays are performed either by an established manual washing procedure or by semi-automated washing based on a 96-needle washer. To further optimize on aspects like reagent consumption, maintenance effort and hands-on time, the assay protocol was adapted to the CYTENA C.WASH™ device. This state-of-the-art washing and liquid handling technology enables easy implementation on 384-well microplates and thereby processes large sample numbers without compromising small sample volume or assay sensitivity and robustness, all while reducing hands-on time by 40%.

a)



b)

Figure 1: *(A) LUNARIS BaseFrame equipped with 4x96-well BioChip. (B) C.WASH Plate Washer and Liquid Dispenser*

LUNARIS Assay performance summary

Sensitivity:
- LOD in single digit pg/mL range with 3-4 log scale quantifiable range

Robustness:
- Uniformity of signal over entire 384-well plate <20%

Performance:
- Within-run Precision <5% CV (9 out of 11 analytes)
- Between-run Precision <10% CV (9 out of 11 analytes) and Accuracy in 80-100% range

Keywords

Immunoassay, Multiplex Analysis, automated plate washing and liquid dispensing, time saving.

Introduction

LUNARIS, AYOXXA´s proprietary beads-on-a-chip multiplex assay platform, enables basic to clinical research from drug development to clinical application[1]. The platform allows simultaneous quantification of up to 12 biomarkers in a format compatible with 384-well microplates **(Figure 1A)**.

The C.WASH **(Figure 1B)**, CYTENA's non-contact plate washer and dispenser, was developed to enhance automated and reproducible washing of multiwell plates. Liquid is removed from microtiter plates using centrifugal forces, resulting in very low residual volumes and a washing efficiency of 99.99% after just two wash cycles[2].

The standard LUNARIS immunoassay protocol includes either manual washing using a multichannel pipette or a semi-automated process using a 96-needle washer. Both protocols deliver sensitivity in single digit pg/mL concentrations with a quantifiable range of 3-4 log scales. However, to meet the requirements of today´s busy life science research labs, a semi-automated washing procedure is most effective at keeping up with the workload without compromising assay robustness.

Homogeneous washing efficiency throughout the immunoassay is essential for the accuracy and precision of the product and for a reproducible assay quality. Here, we describe an adaption of the LUNARIS assay protocol **(Figure 2)** that employs the C.WASH to automate the washing steps and enable high throughput analysis.

Figure 2: *Schematic of the LUNARIS multiplex procedure. (A) The LUNARIS assay follows the classical sandwich ELISA principle. Samples are added to each well of the BioChip and targeted analytes are captured by the antibody-coated beads during incubation. The wells are washed and the captured analytes are labelled with biotinylated detection antibodies and streptavidin-phycoerythrin (SA-PE). The plates are air dried and subsequently imaged with the LUNARIS Reader (B) to quantify the fluorescence signal on the beads. (C) The LUNARIS Analysis Suite Software provides a tailored report with detailed information on the concentration of targeted proteins in each sample.*

Material and methods

To evaluate and optimize the washing protocol, the LUNARIS Mouse 11-plex Chemokine Kit was used (Cat. No. LMCK-20110S or LMCK-20110F). Three LUNARIS BioChips were assembled to the LUNARIS BaseFrame and calibrator curves were set up with a 2.5x dilution factor in Assay Diluent 4, including the analytes CCL2, CCL3, CCL4, CCL5, CCL11, CCL19, CCL20, CCL22, CXCL1, CXCL10 and CX3CL1. Three known concentrations (low,

medium and high) within the quantifiable range of the chemokine standard curves were spiked into mouse serum and used as controls.

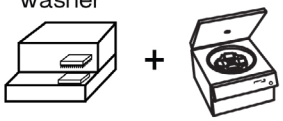
	Reference Method	CYTENA C.WASH
Liquid evacuation set up	96-needle washer + Centrifuge  Aspiration / Reverse centrifugation	 Centrifugal forces
Priming	~300 ml	~40 ml
Pre-wash (wash buffer 1)	1 x 50 µl	1 x 40 µl
Incubation	15 min	15 min
Liquid evacuation	60 sec	10 sec
Sample	5 µl	5 µl
Incubation	*3 h*	*3 h*
Washing (wash buffer 1)	12 x 50 µl	2x 40 µl
Liquid evacuation	60 sec	10 sec
Detection Antibody	10 µl	10 µ l
Incubation	*3 h*	*3 h*
Washing (wash buffer 1)	12 x 50 µl	2x 40 µl
Liquid evacuation	60 sec	10 sec
SA-PE	10 µl	10 µ l
Incubation	*30 min*	*30 min*
Washing (wash buffer 1)	12 x 50 µl	2x 40 µl
Washing (wash buffer 2)	12 x 50 µl	2x 40 µl
Liquid evacuation	60 sec	10 sec
Drying and read out	Dessicator Lunaris reader	Dessicator Lunaris reader
Assay/Hands-on Time (hh:mm)	07:12 / 01:49	06:28 / 01:05

Figure 3: *Schematic of the LUNARIS assay workflow and stepwise assay procedure for the 96-needle reference washer or the C.WASH.*

Throughout the assay protocol, the washing steps were performed with two methods. The first was based on a 96-needle washer and served as the reference method (1 BioChip) **(Figure 2A)** where an efficient elimination of residual liquid is achieved by an additional reverse centrifugation step. The second one is based on the newly established CYTENA C.WASH protocol (3 BioChips) where washing and reverse centrifugation are combined in one step. Details on the workflow are given in **Figure 3**.

The LUNARIS BioChips were prewashed and 5 µL of standard, blank and controls were applied and incubated for three hours at room temperature (RT). After washing the BioChip, 10 µL Detection Antibody Reagent was added and incubated for one hour at RT. For the immunofluorescence detection, the BioChip was washed and subsequently incubated with 10 µL SA-PE Solution for 30 minutes at RT. The plate was air dried and imaged using the LUNARIS Reader 384 **(Figure 2B)** (Cat. No. LRS-202). The quantification of the readout was performed using the LUNARIS Analysis Suite (LAS) software **(Figure 2C)** (Cat. No. LAS-001).

To access reproducibility of the immunoassay, the performance parameter, precision and accuracy among the three BioChips were evaluated. Furthermore, aspects of user convenience and reagent consumption were compared for both washing procedures.

Results and conclusion

The newly established assay protocol for the LUNARIS Mouse 11-plex Chemokine Kit and the C.WASH dispensing and liquid handling system presented an efficient method for semi-automated immunoassay analysis with sensitivities in the single digit pg/mL range and 3-4 log scale quantifiable range.

The standard curve shape for the eleven analytes of the product was comparable between the C.WASH protocol and the reference method. This is indicated by a Coefficient of Variation (CV) <20% for the individual standard points and a CV interreplicate (CVir) for the individual standard points <20% with a mean CVir ranging from 5.5% to 8.3% for the individual analytes **(Figure 4)**.

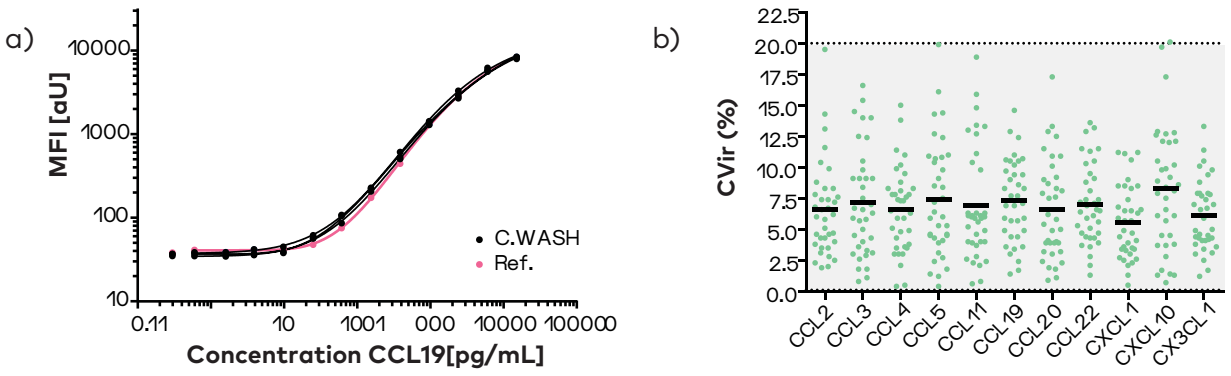


Figure 4: *(A) Exemplary standard curves of CCL19 for the three immunoassays performed with the C.WASH Assay protocol and the immunoassay for the Reference method. (B) Dot plots representing the CVir (%) for the individual standard points and the blank (n=39 for each analyte).*

The summary of assay performance parameters is presented in **Figure 5A**. It also gives a Limit of Detection (LOD) for the eleven analytes in the single-digit range, as well as the lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ), resulting in a dynamic range from 2.4 to 4.0 log-scales.

To determine the recovery for each analyte, the signals measured for the spiked controls were interpolated in the standard curves and correlated to the nominal concentration **(Figure 5B)**. As indicated by the dotted lines, the acceptance criteria are set from 70% to 130%. Nonetheless, all analytes recovered between 80-100%.

a)

	LoD [pg/mL]	LLOQ [pg/mL]	ULOQ [pg/mL]	DR
CCL2	0.8	1.3	800	2.8
CCL3	0.3	0.5	800	3.2
CCL4	0.1	0.2	2000	4.0
CCL5	1.3	2.0	3000	3.2
CCL11	0.7	1.6	960	2.8
CCL19	6.9	9.8	15000	3.2
CCL20	3.0	8.2	2000	2.4
CCL22	3.9	12.3	3000	2.4
CXCL1	1.1	1.6	1000	2.8
CXCL10	2.3	9.8	15000	3.2
CX3CL1	5.3	8.2	5000	2.8

b)

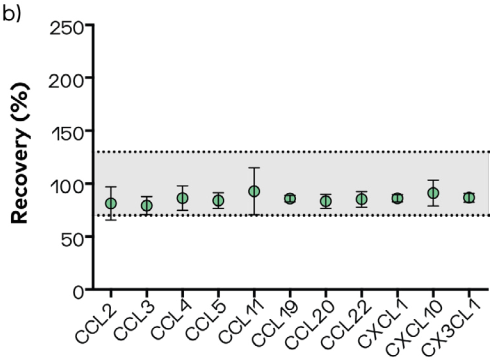


Figure 5: *A) Median assay parameters determined for the three immunoassays performed with the C.WASH-based washing procedure. The limit of detection (LOD), lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) are given in pg/mL; dynamic range (DR) on a log scale. (B) Recovery for the measured QC samples. Each data point represents the mean CV (%) of three QC samples measured in three experiments. The targeted accuracy (70-130%) is denoted by the dotted line.*

Precision of the QC samples spiked into mouse serum was determined as Within Run Precision (WRP), where nine out of eleven analytes performed with <5% CV **(Figure 6A)** and as Between Run Precision (BRP), where nine out of eleven analytes showed <10% CV **(Figure 6B)**.

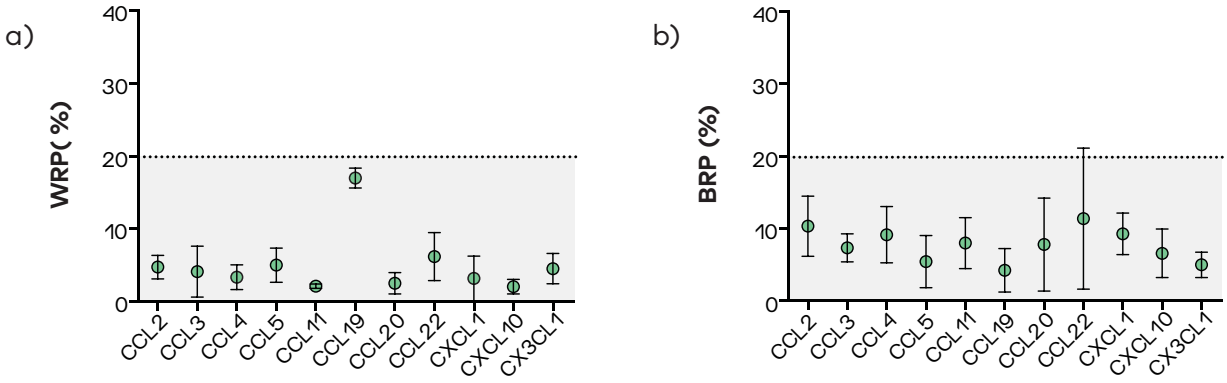


Figure 6: *(A) Within Run Precision (WRP) of QC samples spiked into mouse serum. Each data point represents the mean CV of three QC samples measured in three experiments. The targeted precision (<20%) is denoted by the dotted line. (B) Between Run Precision (BRP) of QC samples spiked into mouse serum. Each data point represents the mean BRP of six different QC sample concentrations measured in three experiments. The targeted precision (<20%) is denoted by the dotted line.*

The four liquid inputs of the C.WASH and the autoprime functionality allowed a straightforward application of different buffers and thereby an easy cleaning routine. The use of centrifugal forces to remove liquid from the plate resulted in a residual volume as low as 100 nL for the spinning intensities used in this workflow. The low residual volumes for each washing step enhanced the washing efficiency per run and reduced the number of wash cycles to two cycles compared to 12 wash cycles in the reference protocol. Considering the full workflow, this reduced the total washing cycles from 48 washes (4x12) to 8 washes (4x2) in total. Furthermore, the C.WASH abolished all reverse centrifugation steps required in the reference method. Overall, these optimizations resulted in a 10% reduction in assay time and a 40% reduction in hands-on time for the assay operator while maintaining the assay quality.

In summary, the combination of LUNARIS Immunoassays and the C.WASH is a powerful tool to speed up the multiplex analysis of biomarkers in a semi-automated process while maintaining the comfort of a benchtop device with reduced reagent consumption.

References

[1]Please find all recent scientific publications using AYOXXA´s LUNARIS™ technology on our website.

[2]Highly efficient washing of microwell plates using centrifugal forces, CYTENA.

Abbreviations

BRP	Between Run Precision
CVir	Coefficient of Variation inter replicate
DR	Dynamic Range
LOD	Limit of Detection
LLOQ	Lower Limit of Quantification
RT	Room Temperature
ULOQ	Upper Limit of Quantification
WB1	Wash Buffer 1
WB2	Wash Buffer 2
WRP	Within Run Precision

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CYTENA spun off from the University of Freiburg, Germany, in 2014 with its patented single-cell dispensing technology. Today, as part of BICO, the world's leading bioconvergence company, CYTENA continues building on that groundbreaking technology to develop high-precision instruments for isolating, dispensing, imaging and handling biological cells. Its award-winning devices are manufactured in Germany and used at prestigious academic and pharmaceutical labs around the world to automate workflows in numerous application areas, including stable cell line development, single-cell omics, high-throughput screening and drug discovery. CYTENA's breakthrough innovations for the lab combine advanced automation, state-of-the-art software engineering and the latest insights in cell biology to maximize efficiencies in the life sciences and create the future of health. Learn more at cytena.com.

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