A novel high performing Multiplex Immunoassay for the Serological Confirmation and Typing of HTLV Infections

Human T-Cell Lymphotropic Virus (HTLV) is responsible for 10 million infections worldwide [1, 2], mainly spread in endemic areas [3, 4, 5]. Infected individuals have a risk of 5-10% of developing one of the two associated diseases [6, 7]. The current standard serological diagnosis has several imitations: lack of specificity [8, 9], elevated costs, and lack of automation [10]. The objective of this project was to develop a new cost-effective Multiplex test to confirm HTLV infection and discriminate the two strains HTLV-1 and HTLV-2. For that purpose, InfYnity Biomarkers has developed a multiplex platform[11, 12] first to identify the most relevant antigens and second to develop a readable and highly performing assay after upgrading the antigen composition with recently identified protein sequences.

Materials and Methods

- *Multiplex Platform*

InfYnity Biomarkers uses sciFLEXARRAYER S3 (SCIENION AG, Germany) to perform the printing of several probes corresponding to different parameters into a 96-well plate format. The printing process is based on piezoelectric pulse through a capillary dispenser. Precision and accuracy of the system enable miniaturization and multiplexing of a standard ELISA into a single well. All probes are printed for specific spatial distribution in all wells. Array configuration of 8 x 7 spots allows multiplexing and miniaturization of the immunoassay in one well.

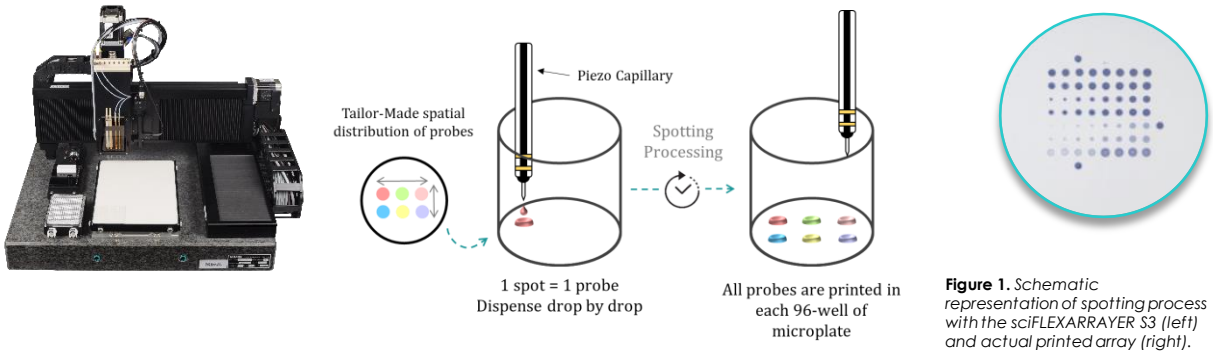

Figure 1. *Schematic representation of spotting process with the sciFLEXARRAYER S3 (left) and actual printed array (right).*

- *Assay design*

Epitope mapping was used to design proprietary antigens specific for different types of target proteins. The final version of the Multi-HTLV is composed of:

- *Three confirmatory antigens for HTLV-1 and HTLV-2 specific antibodies binding. The antigens are derived from common-type and type-specific immunodominant epitopes of env gp21, env gp46 and gag p19.*
- *Three discriminatory antigens derived from type-specific immunodominant epitopes of HTLV-1 env gp46, HTLV-1 gag p19 and HTLV-2 env gp46 antigen*

InfYnity Biomarkers also included in each well positive control spots which are used for the spatial orientation and reagent addition control. Low and medium intensity spots were embedded in each microwell for visual interpretation.

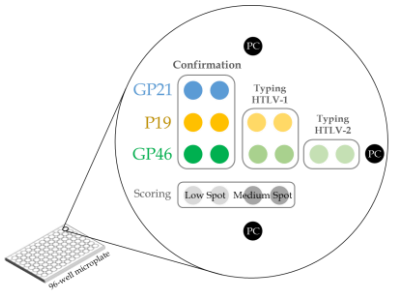

Figure 2. *Schematic description of the Multi-HTLV matrix. This specific targets were spotted in each well of the microplate.*

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(3)Cassar O, Einsiedel L, Afonso PV, Gessain A (2013) Human T-Cell Lymphotropic Virus Type 1 Subtype C Molecular Variants among Indigenous Australians: New Insights into the Molecular Epidemiology of HTLV-1 in Australo-Melanesia.
(4)Mauclère P, Meertens L, Afonso P, et al. HTLV-2 in Central Africa: HTLV-2 subtype B strains similar to those found in Amerindian tribes are endemic in Bakola Pygmies from south Cameroon but not in surrounding Bantus and Baka Pygmies. Retrovirology, 2011
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(7)Casseb J. Is human T cell lymphotropic type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) syndrome a neglected disease?, 2009

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- *Testing process*

The test is performed like a standard ELISA and based on the detection of antibodies. After addition of diluted serum sample, detection is done with an anti-Human IgG-HRP conjugated, and revealed with precipitating substrate.
Each plate was imaged and analysed using the sciREADER CL2 (SCIENION AG, Germany). Medium pixel intensity results were obtained for each spot, see Figure 1.
Based on the intensities, Artificial Intelligence (AI) approach developed especially for this test was applied, in order to obtain a confirmation result, and for positive samples, a typing results.

Figure 3. *sciREADER CL2 for high-quality imaging and multiplex sample analysis*

Results & Discussion

Test performances were evaluated on a characterized cohort of 491 HTLV positive samples from blood donors (including 246 HTLV-1 from 100 patients and 245 HTLV-2 from 100 patients), and 198 negative samples from 198 donors. All samples were confirmed during the construction of the serum collection by both Inno-LIA and PCR; PCR was used as gold standard to evaluate the performance of the Multi-HTLV.
By visual analysis and thanks to the confirmatory antigens, negative and positive results could directly be separated. Further, type 1 and 2 for positive samples could be distinguished. Moreover, the AI method offered the highest performance in terms of sensitivity, specificity and correct classification of HTLV samples.

The developed test is high performing with 100% specificity (198/198 negative donors) and 100% sensitivity (200/200 HTLV positive patients). For positive results, 100% typing correlation with PCR result was obtained.

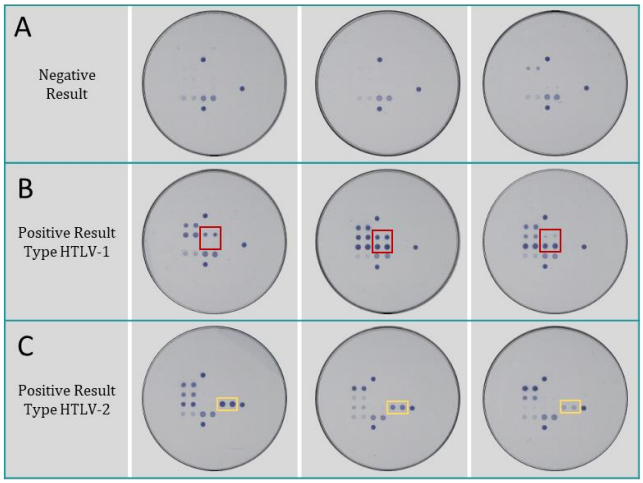

Figure 3. *Examples of different results obtained with Multi-HTLV.*

A) 3 negative samples from blood donors. In most cases no reactivities, is observed but, just one reactive spot. After application of the algorithm, this positive antigen is not associated to a positive result.
B) Three example of positive results typed HTLV-1. which can be typed with the two antigens, but in some cases with only one.
C) Three examples of positive results typed HTLV-2.

Conclusion

With this study, it was demonstrated a highly performing multi-HTLV test that provides different serological patterns per patient. Besides, the high precision AI analysis makes it possible to identify each serological profile and avoid typing errors.
SCIENION technology enables the microplate format for miniaturization and multiplexing, it allows the test to be performed in any routine laboratory with simple equipment and small sample volume. As a result, it provides reduced costs compared to current Immunoblot tests for HTLV confirmation and the acquisition of several answers from a single test, in contrast to the standard ELISA format.
Finally, InfYnity Biomarkers could also show that the Multiplex platform is highly reproducible i.e. assaying identical patients' samples on different production lots showed very small differences in plate-to-plate variability [13].

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