Improved ELISA workflow efficiency using automated plate washing

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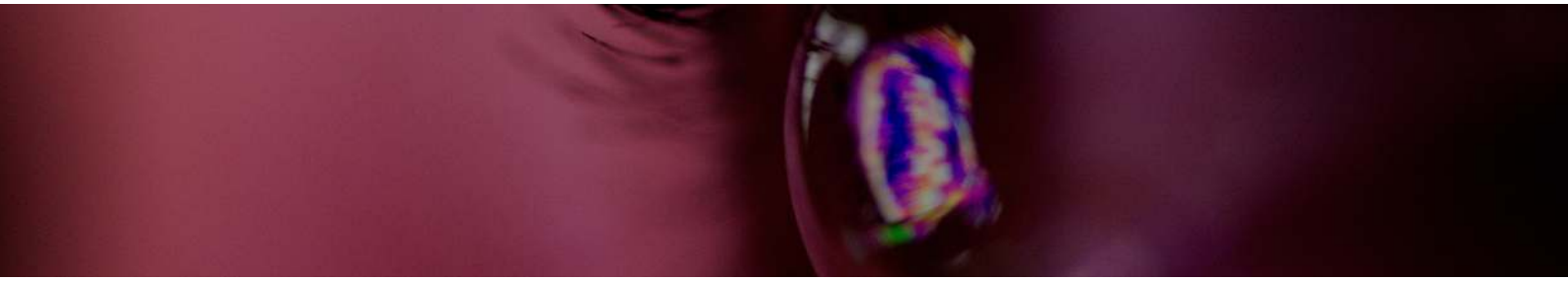

Abstract

Enzyme-linked Immunosorbent Assays (ELISAs) are very sensitive immunoassays, allowing the quantification of specific antigens from complex samples. An essential factor in all ELISA protocols is the washing efficiency as it can directly be correlated to data quality.

The C.WASH, a centrifugal plate washer, enables efficient and reproducible washing of multiwell plates with contact-free dispensing and liquid removal with minimal residual volumes.

A direct comparison between automated plate washing using the C.WASH and manual washing steps was performed using a commercially available ELISA protocol. Utilization of the C.WASH resulted in a tremendous reduction of processing time and costs, and achieves equivalent results.

Introduction

Enzyme-linked Immunosorbent Assay (ELISA) is an analytical method commonly used to quantify the amount of an antigen in solution. The detection is based on the binding of specific antibodies, immobilized on a solid phase. An enzymatic reaction, couped to the antibodies results in a measurable turnover to quantify the amount of antigen in the sample. Potential analytes detected by an ELISA range from diagnostic testing of viral antibodies or secreted proteins in serum or plasma, to toxins or allergens in food samples.

For the evaluation of data quality, the two most important factors are background noise and assay variation. Both of these factors are largely impacted by the washing-efficiency of the protocol. Washing steps within the protocol ensure the removal of unbound and non-specific bound proteins or antibodies that cause unspecific background signals. Typically, all ELISA protocols contain multiple washing steps after each reaction step. Consequently, optimization and automatization of these washing steps holds great potential not only to reduce both time and costs of the workflow but also to increase data quality.

The C.WASH is a non-contact plate washer and liquid dispenser. Liquid can be removed from 96-, 384- and 1536-well plates, based on centrifugal forces. This technology enables fast and reproducible removal of reagents with very low residual volumes. The combination of a non-contact plate washer and liquid dispenser for both 96- and 384-well plates, offers full automation capabilities of all washing steps within an ELISA protocol.

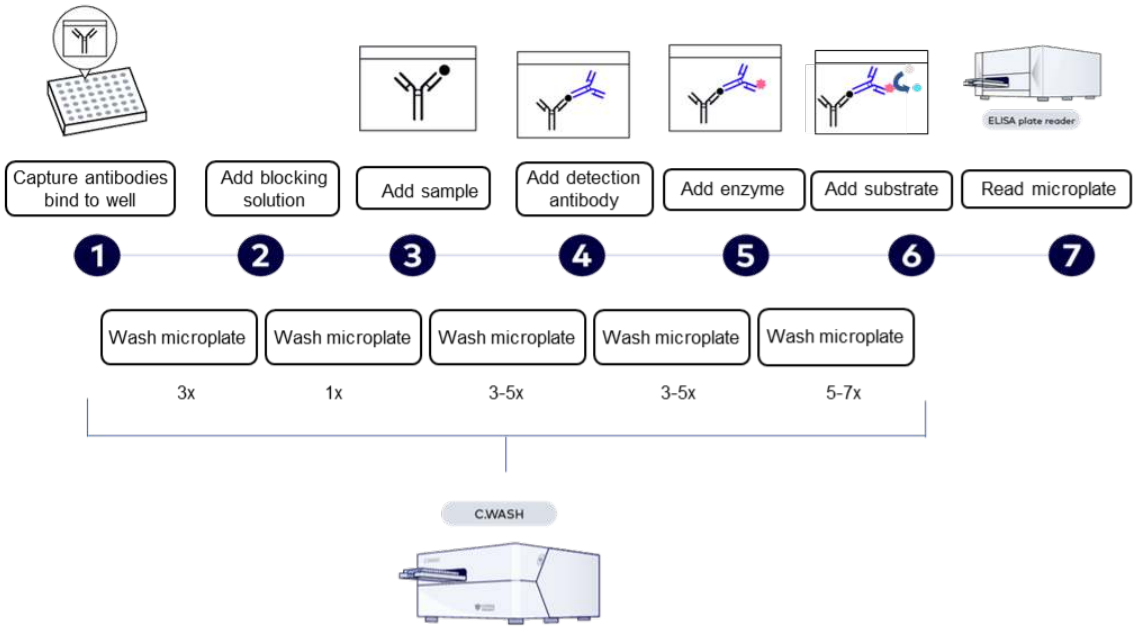

Figure 1. *Workflow of a sandwich ELISA. A microtiter plate is coated with capture antibodies (1) and subsequently blocked (2). The sample is applied (4), followed by detection antibody (4) and a streptavidin conjugated enzyme (5). The addition of substrate (6) will lead to a colorimetric signal analyzed by a microplate reader (7). In between all the reagent additions the microtiter plate is washed, either manually of using the C.WASH microplate washer.*

This application note shows data for a commercially available ELISA Kit to quantify the amount of Tumor necrosis factor alpha (TNF-α) secreted into the cell culture supernatant of stimulated THP-1 cells. Similar results were obtained for Human alpha 1 Antitrypsin ELISA Kit (Abcam, ab108799) and Albumin Human ELISA Kit (Thermo Fisher, EHALBX5) (data not shown).

THP-1 is a suspension cell line, derived from an acute monocytic leukemia patient. This cell line is commonly used as a model cell line for human monocytes. Upon addition of phorbol 12-myristate 13-acetate (PMA) the monocytic cells differentiate into adherent cells with macrophage-like characteristics.

The treatment of those activated THP1 cells with lipopolysaccharide (LPS) mimics a bacterial infection and induces the expression of pro-inflammatory genes. One of the earliest cytokines in the signaling cascade of activated macrophages is TNF-α. This cytokine is involved in almost all inflammatory responses and regulates the activity of immune cells.

Several kits for the detection of human TNF-α are commercially available. The kit used in this experiment relies on the workflow of a sandwich ELISA, shown in **Figure 1**.

1. A solid surface, typically the well bottom of a microtiter plate, is prepared by immobilizing capture antibodies.
2. After 3 rounds of washing, a blocking solution is added to avoid any nonspecific binding on the surface.
3. After another wash cycle the sample is added and binds to the capture antibody. All unbound antigen is subsequently removed by 3-5 washing cycles.
4. A biotinylated detection antibody is added. This antibody is specifically recognizing and binding the TNF-α antigen. Afterwards the microplate is washed 3-5 cycles.
5. Streptavidin-horseradish peroxidase (HRP) conjugates are added, coupling the enzyme to the detection antibody. All unbound enzyme conjugate is removed by 5-7 wash cycles.
6. HRP substrate 3,3′,5,5′ tetramethylbenzinidine (TMB) is added. The substrate is oxidized by immobilized HRP results in a blue colored solution. The intensity of color correlates with the concentration of detected antigen.
7. The microplate is analyzed by a colorimetric measurement using a microplate reader.

In this work we compared automated washing steps, as performed by the C.WASH, to manually performed washing steps, by following the same commercially available protocol.

Materials and methods

THP-1 cells were differentiated with 500 nM phorbol 12-myristate 13-acetate (PMA, PeproTech, 1652981) for 24 hours and subsequently TNF alpha production was induced by the addition of 1 µg/mL lipopolysaccharide (LPS, eBioscience™ Lipopolysaccharide (LPS) Solution (500X), Invitrogen™, 00-4976-93) for 48 hours. The cell culture supernatant was recovered and used for analysis in the ELISA assay.

The protocol of the TNF alpha Human Uncoated ELISA Kit (Invitrogen) was adapted for use in 384-well plates by decreasing the volumes for sample and reagents to 30 µL per well, and the volume of wash buffer in the washing steps to 100 µL per well. All washing steps were performed using PBS wash buffer (PBS, 0.05% Tween-20).

A total of 15 washing steps were performed in the protocol. These washing steps were in parallel processed manually and using the C.WASH. The washing steps using the C.WASH were performed at a spinning intensity of 150 g.

A solution of sulphuric acid (2 N) was used as a stop solution to stop the HRP activity after 15 minutes reaction time. Absorption was measured at 450 nm and 570 nm to compensate for optical interference.

Results and discussion

For both manual washing and automated washing in the C.WASH, the resulting TNF-α concentrations are very comparable, as shown in Figure 2. TNF-α concentration were measured for cell culture supernatant (a), as well as for 2 different concentrations of the provided TNF-α standard (b and c). For each condition, a column of 16 wells, from A to P, was analyzed. Figure 3 shows the calibration curves for both the manual and the automated protocol. Both curves are comparable and result in an R^2 value of 0,999.

Calculated signal/noise ratios are equivalent for manual washing steps and automated washing in the C.WASH.

The main advantage of using the C.WASH for ELISA wash steps is the saving in both time and consumables.

Using the tipless dispensing system of the C.WASH, all washing steps were performed without the use of any pipette tip. Assuming a fresh tip is used for each well to manually remove the reagent/wash buffer, and a set of 12 tips is used for each dispensing of the washing buffer, that adds up to 396 tips per wash and 5940 tips over the full ELISA protocol for one 384well microtiter plate.

Table 1. *Comparison of time and pipette tip consumption in both protocols.*

		Manual	C.WASH
Time	1 wash cycle	~3 min	40 sec
	15 wash cycles	45 min	10 min
Pipette tips	1 wash cycle	396	0
	15 wash cycles	5940	0

Figure 2. *TNF-α concentrations measured in the assay with manual washing steps (blue) and using the C.WASH (green). Each measurement was performed in one column of the plate (A to P) to access the variability across the plate. A) cell culture supernatant from THP-1 cells, B) and C) different concentrations of TNF-α standard solution*

The time needed for one wash cycle in the C.WASH is 40 seconds for a full 384 well plate. The residual volume at the spinning velocities used in this protocol is below 100 nL. Manually removing the liquid, including tapping to ensure low residual volume and the addition of fresh washing buffer manually, is at least 4-fold slower (~3 min). The time saved on washing steps multiplies with the 15 washing steps, results in a drastic reduction of total protocol time for automated plate washing using the C.WASH.

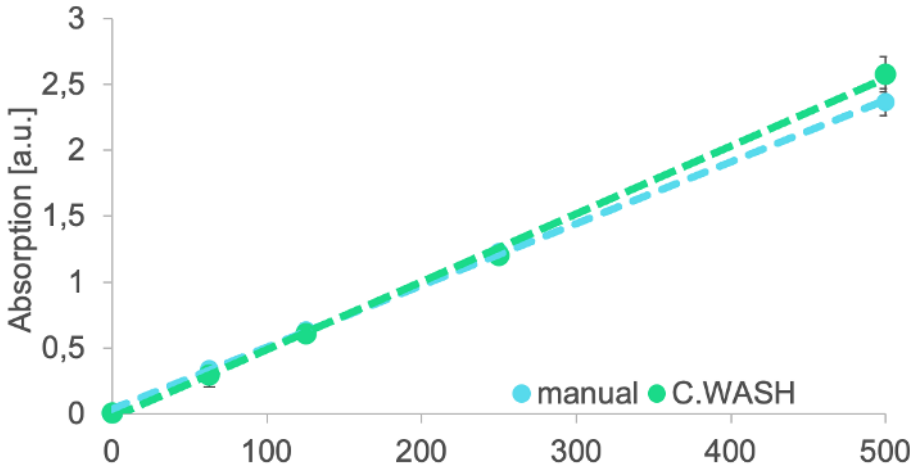

Figure 3. *Calibration curve for TNF-α standard solution in the manual protocol (blue), compared to the automated washing using the C.WASH (green). Data shown are mean values deriving from measurements in triplicates.*

Conclusion and future direction

- The C.WASH reduces both the time and cost required to perform ELISA washing steps, when compared to conventional manual methods
- Higher reproducibility and consistent data quality is achieved by automating the washing steps

References

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