Automation of three ELISA protocols on the Opentrons OT-2 with an on-deck Heater-Shaker Module

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ABSTRACT

Enzyme immunoassays (EIAs) or enzyme-linked immunosorbent assays (ELISAs) are widely used analytic tools for detection of a target molecule (e.g., an antigen) in liquid samples for diagnostic or research purposes. The assay is usually designed in 96-well format, and the workflow generally includes reagent transferring and mixing, several incubations at a set temperature and washing steps between incubations. All these steps require a high level of consistency in sample handling. To utilize Opentrons OT-2 liquid handler and the Heater-Shaker Module to perform this popular quantitative method, fully automated protocols were developed and tested using Takara Bio's sandwich EIA for detection of human fibronectin (FN), Tecan's competitive ELISA for saliva Cortisol detection, and another competitive ELISA from Cell Sciences to quantify neutralizing SARS-CoV-2 antibody in serum samples.

Key findings

- The OT-2 equipped with an 8-Channel Pipette and a Heater-Shaker Module demonstrated the ability to conduct ELISAs with excellent precision.
- In a higher throughput setting, this protocol can handle up to 96 samples with minimal hands-on time.

INTRODUCTION

Enzyme immunoassays (EIAs) or enzyme-linked immunosorbent assays (ELISAs) refer to a technique to detect and quantify a target molecule (e.g., an antigen) in a liquid sample through highly specific antibody-antigen interactions. This assay is often designed to take place in a 96-well or 384-well polystyrene plate, which provides the solid surface for immobilization of the antigen, achieved by directly attaching the antigen to the solid surface or via the use of a capture antibody pre-coated on the solid surface. Through this process, it facilitates the separation of the antigen from the rest of the non-target molecules in the sample.

The sandwich EIA (or ELISA) is the most used format of the assay. The procedure usually involves following steps:

1. Attach the capture antibody (i.e., an antibody specific to the target of interest) to the plate (e.g., 8-well or 12-well stripes assembled on a frame compatible to a 96-well microplate reader) by passive adsorption if the plate pre-coated with that antibody is not supplied by the vendor
2. Add samples to be analyzed to the antibody-coated plate allowing target antigen to be captured
3. Expose immobilized target antigen to the detection antibody conjugated with an enzyme (e.g., horseradish peroxidase or HRP)
4. Add a substrate for the enzyme to produce a signal that can be measured and quantified

The human fibronectin (FN) EIA kit (Takara Bio, San Jose, CA, USA) is an in vitro assay kit for quantitative determination of soluble human FN in serum, urine, cell culture supernatants, and other biological fluids. The kit provides sufficient reagents and materials for EIA protocol design and validation on the OT-2. FN is widely distributed on cell surfaces, in the extracellular matrix, and in plasma, involved in cell-to-substrate adhesion, cell migration, regulation of cell morphology, and other cellular functions. Takara's FN EIA kit utilizes two mouse monoclonal anti-human FN antibodies allowing an antibody-antigen-antibody sandwich to form: the capture antibody pre-coated on 8-well strips, and the detection antibody conjugated with peroxidase. The photometric signal can be developed by exposing this complex to a substrate of peroxidase, with the absorbance proportional to the amount of target (i.e., human FN) present in the sample.

Competitive ELISAs measure the concentration of an antigen by detection of signal interference. Briefly, the target antigen competes with a reference antigen, which is pre-coated on the plate, for binding to the enzyme-conjugated antibody. The more the target antigen is present in the sample, the less the reference antigen can bind the antibody and the weaker the signal produced. In some competitive ELISA kits, such as Tecan's Cortisol Saliva ELISA (Männedorf, Switzerland) tested in this study, instead of the antibody, the enzyme is carried by the reference antigen which competes with the target antigen for antigen-antibody interaction. Another ELISA validated on the OT-2 was SARS-CoV-2 Surrogate Virus Neutralization Test kit (Cell Sciences, Newburyport, MA, USA) measuring circulating neutralizing antibodies produced after SARS-CoV-2 infection or in response to a vaccination. Neutralizing antibodies inhibit the entry of SARS-CoV-2 into cells by blocking the binding of angiotensin converting enzyme 2 (ACE2) and receptor-binding domain (RBD). The kit provides a pre-coated plate of RBD of the viral spike protein and utilizes the format of a competitive ELISA by introducing HRP-conjugated ACE2 to compete with the neutralizing antibody for capturing RBD.

Despite the variety of assay design, ELISAs typically follow a similar workflow. The general steps such as reagent transfer, incubations, and repeated washes required to complete the assay can be easily automated on the OT-2. Serial washes are performed during this process to remove unbound molecules between assay steps. To prevent the remaining solution carried over to the next assay step, it is essential that excess liquid is aspirated completely. Opentrons 8-Channel Pipettes can perform precise and accurate liquid handling to fulfill the needs of reagent transfer and washing in ELISAs. In addition, this assay can be fully automated with the Opentrons Temperature Module or Heater-Shaker Module integrated onto the OT-2 platform to provide set temperature for sample incubation, for example at 37°C to promote antigen-antibody interaction.

MATERIAL AND METHODS

Schematic of three ELISAs tested on the Opentrons OT-2, the outline of steps to compete the assay, and the deck layout (Figure 1-3)

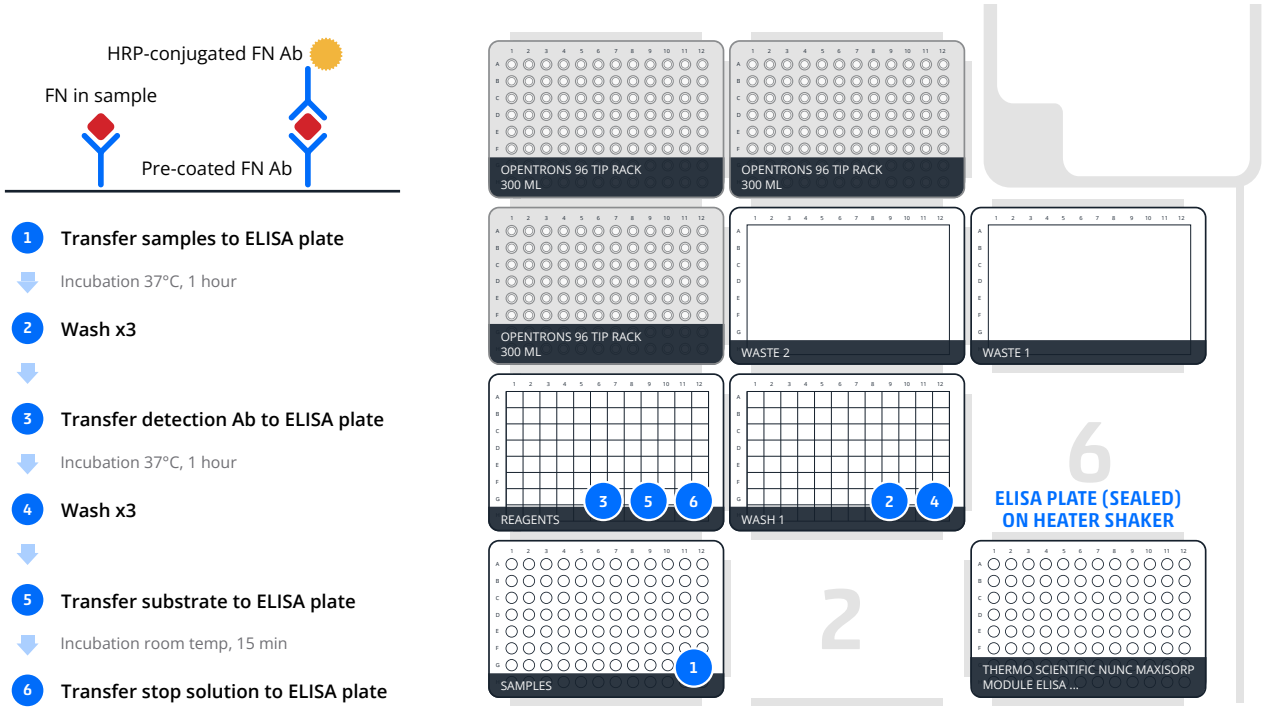


Figure 1: Takara's human FN sandwich EIA performed on the OT-2

1. **Transfer samples to ELISA plate**
2. **Transfer Ag with HRP to ELISA plate**
   Incubation room temp/shaking, 2 hours
3. **Wash x4**
4. **Transfer substrate to ELISA plate**
   Incubation room temp/shaking, 0.5 hour
5. **Transfer stop solution to ELISA plate**

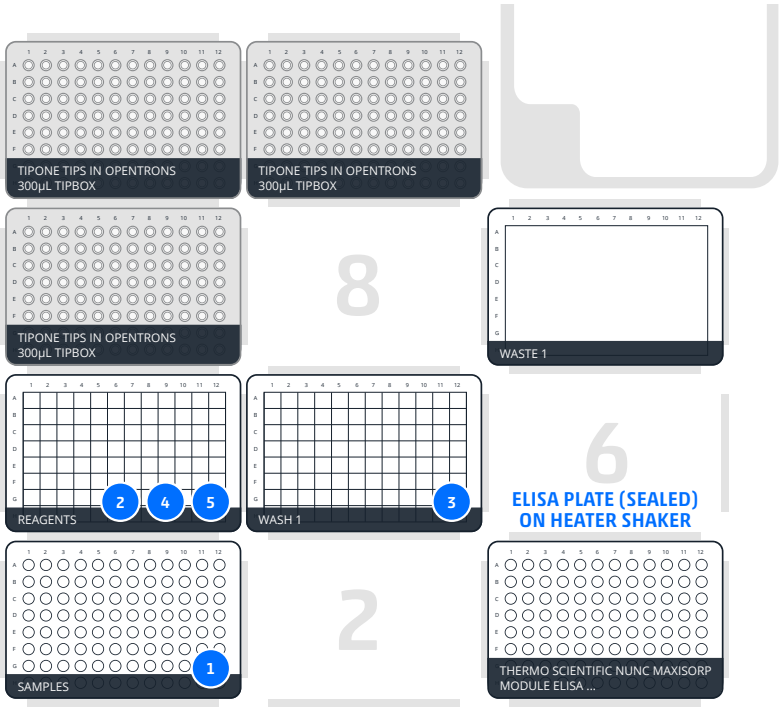
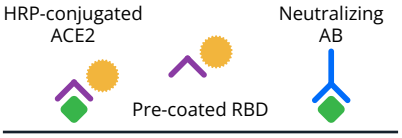


Figure 2: Tecan's cortisol saliva competitive ELISA performed on the OT-2



1. **Transfer samples to ELISA plate**
   Incubation 37°C, 0.5 hour
2. **Transfer RBD with HRP to ELISA plate**
   Incubation 37°C, 1 hour
3. **Wash x3**
4. **Transfer substrate to ELISA plate**
   Incubation 37°C, 10 min
5. **Transfer stop solution to ELISA plate**

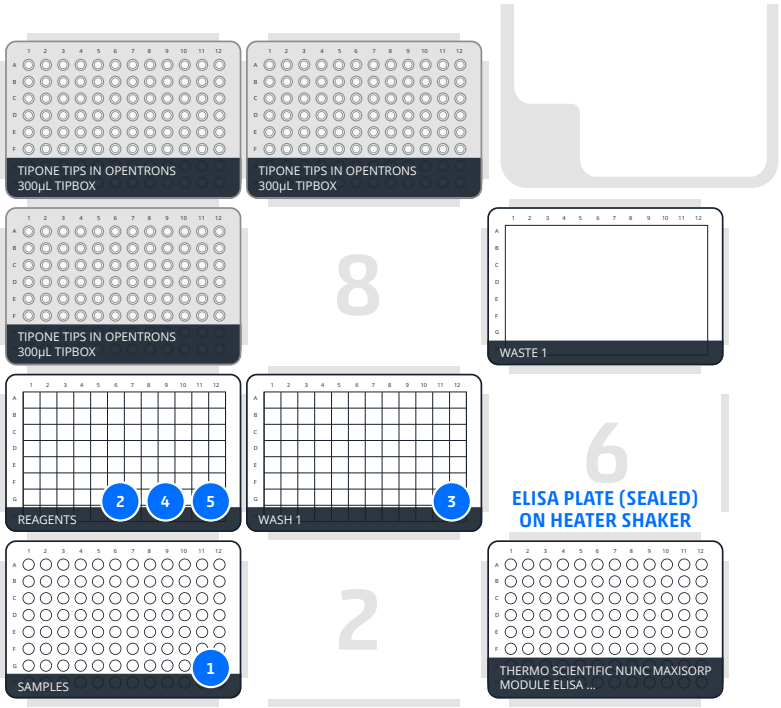


Figure 3: Cell Sciences' SARS-CoV-2 surrogate virus neutralization test (competitive ELISA) performed on the OT-2

Liquid transfer was performed by the 8-Channel Pipette. The ELISA plate was sealed with a slit seal (BioChromato, Fujisawa, Japan) which allows pipet tips to penetrate while preventing evaporation during incubation and then latched on the Heater-Shaker Module, which provides heat and agitation if requested per instructions **(Table 1)**. Immediately after the assay was completed, the ELISA plate was moved manually to a photometric microplate reader and absorbance measured at 450 nm.

To test FN EIA kit, samples were prepared by dissolving human plasma FN in deionized water to make a stock solution of 1 mg/mL and further diluted to 500 ng/mL and serial 2-fold dilutions. To test cortisol ELISA, the standards and control solution with known cortisol concentrations provided in the kit were used. Serum samples previously tested as SARS-CoV-2 antibody positive or negative were obtained for virus neutralization ELISA. The runtime for each assay at different sample sizes was estimated **(Table 1)**.

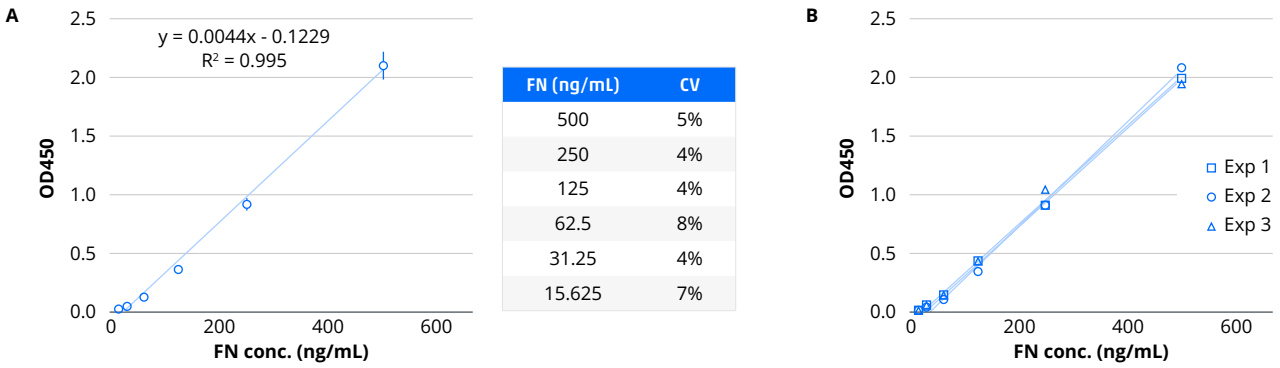
RESULTS

FN EIAs were processed on the OT-2. The quality of sample handling can be evaluated by assessing the correlation between the final readout and the concentration of the target molecule and the consistency of this correlation between assays. The goodness-of-fit for linear regression was determined by plotting the average absorbances (n=3) against the concentrations of FN, and standard deviations calculated. The results demonstrated the accuracy and precision of OT-2 on performing this assay kit (R-squared > 0.99, CV < 10%) **(Figure 4A)**, and the reproducibility of the assay was also confirmed **(Figure 4B)**.

Serial dilutions of cortisol were used to test a competitive ELISA on the OT-2. The results demonstrated the consistency of liquid handling (n=3, CV < 10%), an excellent negative linear association of the assay readout versus log10-transformed sample concentration on a semi-logarithmic plot (R-squared > 0.99) **(Figure 5A)**, and reproducibility between assays **(Figure 5C)**.

Table 1: Heater Shaker Module settings for each assay and the estimated runtime at different sample sizes

		TAKARA BIO FN EIA	TECAN CORTISOL SALIVA ELISA	CELL SCIENCES SARS-COV-2 SURROGATE VIRUS NEUTRALIZATION TEST
Assay format		Sandwich ELISA	Competitive ELISA	Competitive ELISA
Heater Shaker settings				
Incubation (target/ reference antigen capturing)	Shaking (rpm)	500	500	500
	Time (min)	0.2	120	5
	Heating (°C)	37	Room temp	37
	Time (min)	60	120	90
Incubation (detection antibody binding)	Shaking (rpm)	500	-	-
	Time (min)	0.2	-	-
	Heating (°C)	37	-	-
	Time (min)	60	-	-
Signal development	Shaking (rpm)	500	500	500
	Time (min)	0.2	30	0.2
	Heating (°C)	Room temp	Room temp	37
	Time (min)	15	30	10
Estimated runtime				
24 samples (3 columns)		2.7 hrs	2.8 hrs	1.9 hrs
48 samples (6 columns)		3.2 hrs	3.1 hrs	2.2 hrs
96 samples (full plate)		4.1 hrs	3.6 hrs	2.7 hrs



FN (ng/mL)	CV
500	5%
250	4%
125	4%
62.5	8%
31.25	4%
15.625	7%

Figure 4: Takara's human FN sandwich EIA was successfully performed on the OT-2 with highly consistent sample handling (A, CV obtained by processing each concentration in triplicate) and reproducible results between 3 separate tests (B)

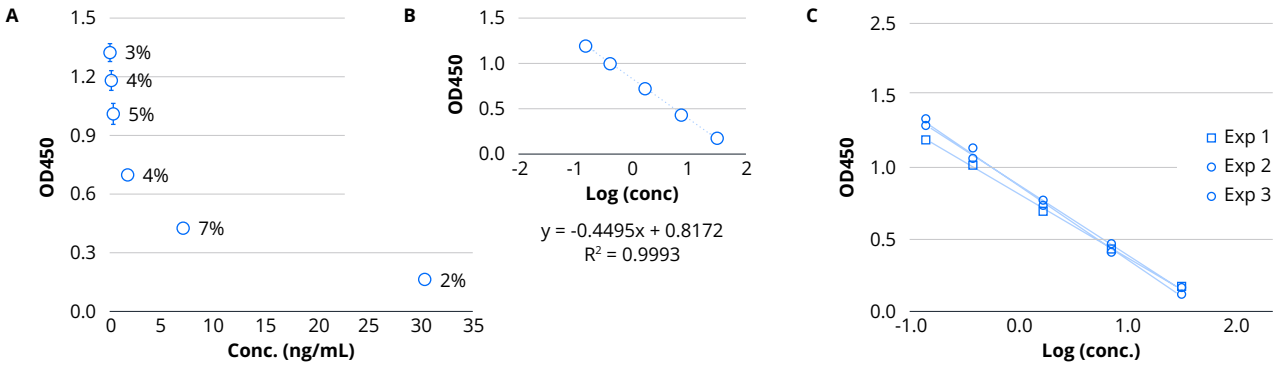


Figure 5: Highly consistent results were obtained by processing serial dilutions of cortisol in triplicate on the OT-2 for Tecan's cortisol saliva competitive ELISA (A). The negative linear association of the assay readout versus log10-transformed sample concentration on a semi-logarithmic plot (R-squared > 0.99) was obtained (B). The reproducibility was confirmed by comparing 3 separate assays (C).

To detect inhibition of ACE2-RBD binding by a competitive ELISA, serum samples were processed on the OT-2 in triplicate, absorbance measured on a plate reader, and CV obtained. The results again confirmed the precision of OT-2's liquid handling (CV < 10%) **(Figure 6)**. Per the manufacturer's instructions, the assay was valid as the OD450 value of each control fell within the range (< 0.3 for positive control and > 0.9 for negative control). Inhibition rate was calculated by:

Inhibition rate = [1 –(OD450 value of sample/ OD value of negative control)] x 100%

The presence or absence of neutralizing antibodies in each sample was determined by comparing its inhibition rate to the positive cutoff (>= 20%) or negative cutoff (<20%). The assay successfully detected the antibodies that neutralize the ACE2-RBD interaction in the samples previously diagnosed as SARS-CoV-2 antibody positive **(Table 1)**.

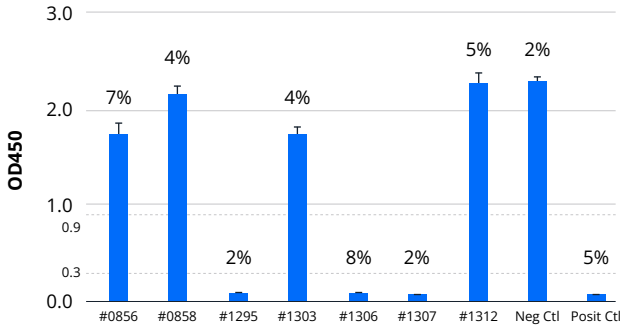


Figure 6: Cell Sciences' SARS-CoV-2 surrogate virus neutralization test performed on the OT-2 measured neutralizing antibodies present in the human serum samples with excellent precision. The assay was valid as the OD450 value of each control fell within the range (< 0.3 for positive control and > 0.9 for negative control).

Table 2: Human serum samples that had previously tested positive by FDA-certified ELISA (*) were confirmed by the assay (inhibition > 20%)

	INHIBITION	
#0856	24%	*
#0858	6%	
#1295	97%	*
#1303	23%	*
#1306	96%	*
#1307	97%	*
#1312	1%	

CONCLUSION

Three ELISA assays, a sandwich ELISA, a competitive ELISA detecting an antigen, and a competitive ELISA detecting an antibody, were successfully automated on the OT-2 . The results demonstrated an excellent quality of sample handling comparable with conventional manual approaches.