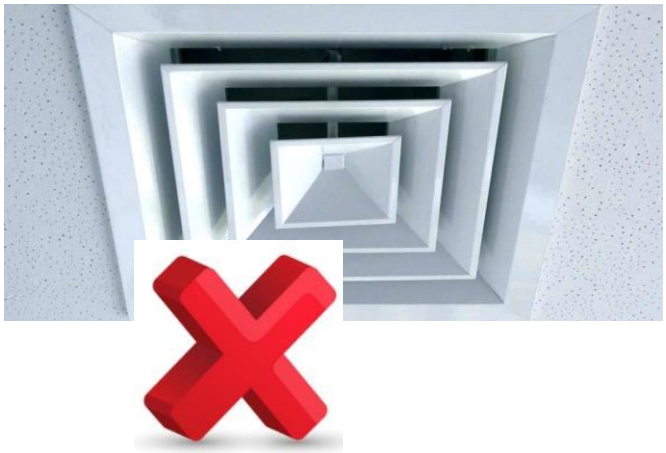
GETTING STARTED WITH MICROFLUERE® 96-WELL ELISA MICROPLATE

Although MicroFluere® 96-well ELISA microplate is fully compatible with most of the existing ELISA equipment, there are 2 key activities that will make the transition to using MicroFluere® easy and rewarding. These steps should not take more than 1-2 hours of your time. Also please review the following video - https://www.optobio.com/wp-content/uploads/2021/04/Drainage-device-1.mp4:

Practice pipetting without introducing bubbles in the microfluidic channels.

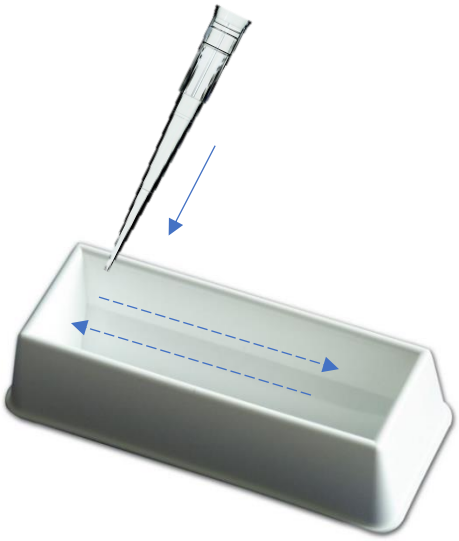
1. Before starting, make sure the environment is free of any fans that could cause evaporation. After every step make sure to put plates in a sealed container or drawer or box. Evaporation is quick with small volumes of liquids and can drastically affect concentration.



2. When mixing solutions there are two groups. One (for standard, detection, capture, wash, and reagent diluent) we recommend pipetting up and down 5 times. DO NOT use the vortex machine to mix for this group. The other group (for HRP and Substrate) it is okay to use a vortex machine to mix. When extracting liquid using a pipette, make sure the tip is below the top surface. This will ensure no air bubbles are pulled up. (See the following videos: https://www.optobio.com/wp-content/uploads/2021/04/Pipetting.mp4 and https://www.optobio.com/wp-content/uploads/2021/04/Pipetting2.mp4).

3. Make sure you have enough volume of liquid for each step. It is recommended to go over the expected amount. For example, **500 µl** of standard for each column was prepared even though **160 µL** was needed (8 channels*20ul/channel = **160 µL**).

4. To mix solutions in a multichannel pipette reservoir, make sure the proper amount of buffer fully covers the bottom of the reservoir. Next, dispense the required amount of antibody at one end of the reservoir, and slowly move the pipette down the length of the reservoir slowly pipetting up and down to mix the solution without introducing air bubbles. This ensures that the multichannel pipette does not pick up any bubbles.

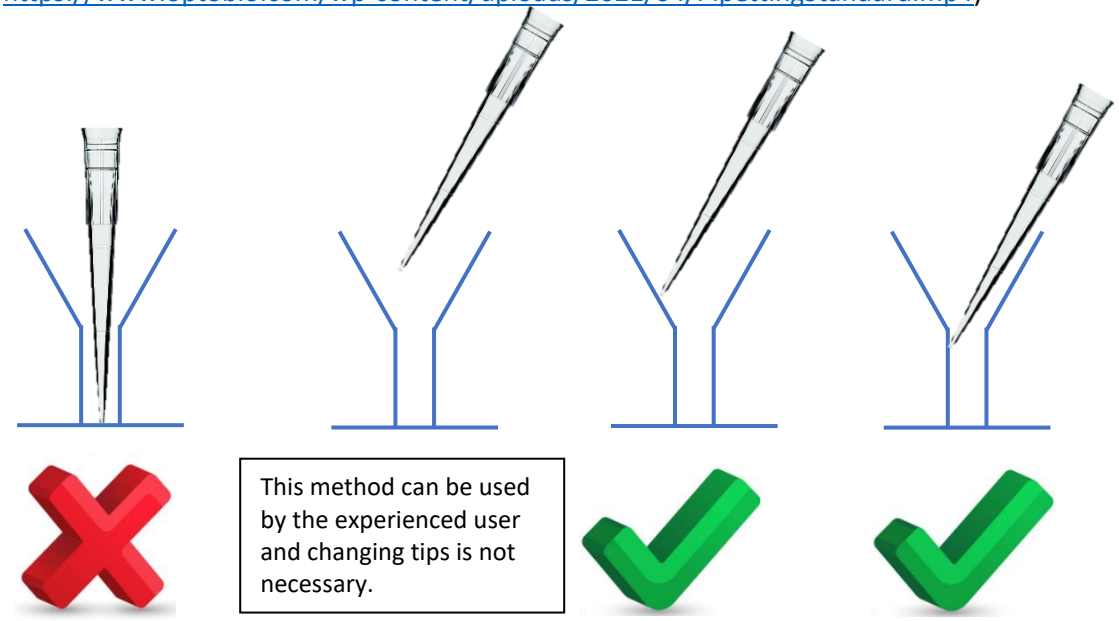


5. Standard solutions can be prepared via serial dilution in tubes where the concentration from one tube to the next is reduced by 50% (or user defined). Use only one pipette tip at a fixed volume during the serial dilution. Mix gently by pipetting ~5 times after each transfer and be careful to not introduce air bubbles. This will ensure there is no loss of signal without having to switch tips between each standard. If using a multichannel pipette, transfer or initially make the solutions in 0.5 mL tubes as shown in figure below (see the following video: https://www.optobio.com/wp-content/uploads/2021/04/StandardCurve.mp4).

Serial Dilution Scheme
0.5 mL tubes

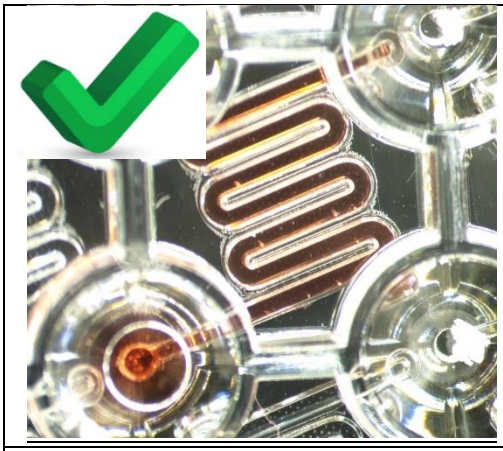
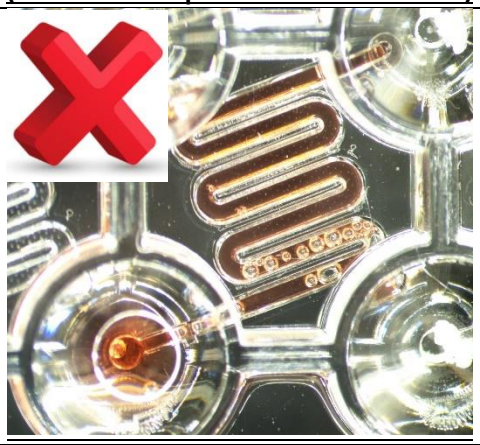
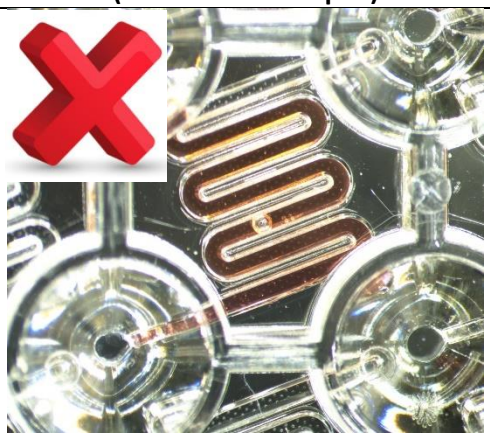
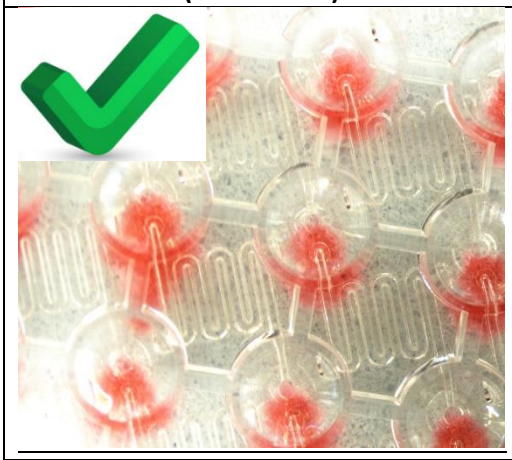
6. When pipetting into the channels, make sure all pipette tips are aligned over the microchannel openings. Make sure to avoid touching the film with the tip though the opening (see 1st left schematic) that may result in leakage across channels. You can start with pipetting methods as described in 3rd and 4th schematics. Go as far down into the opening as you can without touching the film (see far right 4th schematic) or touch the tip to the funnel (see 3rd schematic). For the first two steps (capture and blocking), the user can simply touch the pipette tip to the funnel or slightly below of the funnel without changing pipette tip each time. After standards, the pipette tips must be changed (1 pipette tip/well). When you become an experience user, you can use the 2nd pipetting method (Tip is not touching and just hanging over the microchannel openings.) to avoid changing pipette tip each time.

   Note: When dispensing small volumes into the channels, it helps to have a quick, smooth, and continuous motion with the pipette. Only go to the first stop on the pipette; **never try dispensing the additional liquid after the first pipette stop, as this likely will introduce air bubbles** (see the following video: https://www.optobio.com/wp-content/uploads/2021/04/PipettingStandard.mp4)



7. Complete and proper draining after each step is more important than rushing to the next step. **The non-experienced user should routinely check all the microchannels after each drainage step and familiarize themselves with proper and improper filling and draining.** To visually inspect the plate after draining, flip it over and place against a dark background to inspect the channels for any liquid residual. It is easiest to find trapped liquids by slightly rotating the plate in the light. If necessary, repeat the draining process. We also recommend using two times daring (use new absorbent pad each time) each step (except washing steps) to eliminate cross contamination from one step to another.

8. **Example photos**

	
Proper filling **(Continuous liquid without air bubble)**	**Improper filling** **(Discontinuous liquid)**
	
Improper filling **(Air bubbles)**	**Improper filling** **(Discontinuous liquid and an air bubble)**
	
Proper draining **(No liquid remaining in the channels.)**	**Improper draining** **(Some liquid remains in the channels.)**

Prepare the plate reader for Chemifluorescent readings

1. **Substrate**: MicroFluere® was tested and verified only with a Chemifluorescent substrate from ThermoFisher Scientific (QuantaRed™ Enhanced Chemifluorescent HRP Substrate Kit, Cat. # 15159 https://www.thermofisher.com/order/catalog/product/15159#/15159.). Therefore, the user needs to have it for MicroFluere® ELISA experiments.

2. **Microplate reader**: MicroFluere® is designed to fit with any microplate readers which can read traditional 96-well plate with fluorescent detection mode in either top or bottom reading option. Compatibility of MicroFluere® has been tested with following plate readers.
    - EnSpire 2300 Multimode Plate Reader (PerkinElmer)
    - Infinite F200 Fluorescent Microplate Reader System (Tecan)
    - Spark® Multimode Microplate Reader (Tecan)
    - Synergy Neo2 Hybrid Multi-Mode Reader (BioTek Instruments)
    - Synergy H1 Hybrid Multi-Mode Reader (BioTek Instruments)
    - Synergy HT (BioTek Instruments)
    - Synergy HTX Multi-Mode Reader (BioTek Instruments)
    - SpectraMax M2 (Molecular Devices)
    - SpectraMax M2e (Molecular Devices)
    - SpectraMax iD3 (Molecular Devices)
    - GloMax®-Multi+ Detection System (Promega)
    - Varioskan Flash (Thermo Fisher Scientific)

In general, there are three different types of fluorescence microplate readers as follows:

(1) monochromator-based plate readers with adjustable measurement height: tunable wavelengths (excitation and emission) with adjustable Z-height of the plate (distance between the objective lens of the plate reader and the well plate)
(2) filter-based plate readers with adjustable measurement height: specified wavelengths (excitation and emission) optical filter set with adjustable Z-height of the plate (distance between the objective lens of the plate reader and the well plate)
(3) filter-based plate readers: specified excitation and emission wavelengths optical filter set (without Z-height adjustment option)

Selection of excitation and emission wavelengths: If the reader is monochromator-based, set the excitation and emission at ~570 and 585 nm which are absorption and emission maxima of fluorescent reaction product of the substrate. Quantitation does not require precise matching of the excitation/emission maxima. If the reader is filter-based, select a filter set of 530-575 nm for excitation and 585-630 nm for emission.

Plate reader quick assessment:

a. Prepare fluorescence solution using QuantaRed™ substrate mixed with horseradish peroxidase (HRP) to convert it to fluorescence solution. Add the stop solution.
b. Dispense 100 μL of the fluorescence solution to a well of traditional 96-well plate.

c. Read two wells (with and without the fluorescence solution) of the 96-well plate using the excitation and emission wavelengths described above.
d. Adjust excitation light intensity (e.g., number of flash) and collection efficiency (e.g., gain) at standard Z-height to achieve the lowest background signal from the empty fluorescence well and the highest signal from the fluorescence solution filled well. The difference between the background signal and fully developed substrate should be about 3 orders of magnitude (at least 100 times depending on model of plate reader).
e. **Adjust Z-height, if available option:** Detection Z-plane of the MicroFluere® is above that of the traditional well plate. If the reader has adjustable Z-height option for the plate, adjust it 3-5 mm away from the traditional well plate setting (i.e., based upon top or bottom reading, the Z-height should be decreased or increased, respectively).
f. Repeat steps a-d above with the MicroFluere microplate, adjusting the Z-height (If the plate reader has an option to adjust Z-height) to achieve maximum detection efficiency.
g. Now, the plate reader is ready for MicroFluere® experiments.

Examples of key settings of plate readers:

(1) EnSpire 2300 Multimode Plate Reader (PerkinElmer)

Using of excitation filter	Top
Number of flashes	100
Excitation wavelength	570 nm
Emission wavelength	590 nm
Measurement height	13 mm (whereas 6.1 mm for traditional 96-well plate)

(2) Synergy Neo2 Hybrid Multi-Mode Reader (BioTek Instruments)

<u>Top reading option</u>

Optics:	Top, Gain: 40
Excitation:	530/25
Emission:	590/35
Light Source:	Xenon Flash, Lamp Energy: Low
Read Speed:	Normal, Delay: 0 msec, Measurements/Data Point: 10
Read Height:	10.25 mm (whereas 5 mm for traditional 96-well plate)

<u>Bottom reading option</u>

Optics:	Bottom, Gain: 40
Excitation:	530/25
Emission:	590/35
Light Source:	Xenon Flash, Lamp Energy: Low
Read Speed:	Normal, Delay: 0 msec, Measurements/Data Point: 10

(3) Synergy HTX Multi-Mode Reader (BioTek Instruments)

<u>Top reading option</u>

Optics:	Top, Gain: 53 (or Automatic)
Excitation:	560/20
Emission:	610/10
Read Height:	**<u>4.5 mm</u>**

Bottom reading option

Optics:	Top, Gain: 47 (or Automatic)
Excitation:	560/20
Emission:	610/10

(4) Infinite F200 Fluorescent Microplate Reader System (Tecan)

Plate	Greiner 96 Flat Bottom Transparent Polystyrol
Mode	Fluorescence Top Reading
Excitation Wavelength	560 nm
Emission Wavelength	612 nm
Excitation Bandwidth	20 nm
Emission Bandwidth	10 nm
Gain	50 Manual
Number of Flashes	25
Integration Time	20 µs
Lag Time	0 µs
Settle Time	0 µs

(5) Spark® Multimode Microplate Reader (Tecan)

Plate	Greiner 96 Flat Transparent
Mode	Fluorescence Top Reading
Excitation Wavelength	570 nm
Excitation Bandwidth	5 nm
Emission Wavelength	600 nm
Emission Bandwidth	5 nm
Gain	160 Manual
Mirror	50% Mirror
Number of Flashes	30
Integration Time	40 µs
Lag Time	0 µs
Settle Time	0 µs
Z-Position	2250 µm
Z-Position mode	Manual

(6) SpectraMax M2 or M2e (Molecular Devices)

Top reading option

Plate	96 Well Costar clear
PMT and Optics:	Medium, 6 Flashes/read
Excitation:	560 or 570
Emission Cutoff	**590**
Emission:	610

(7) SpectraMax iD3 (Molecular Devices)

Top reading option

Plate	96 Well Falcon clear
PMT Gain:	Manual, 500 volts
Integration Time:	10 ms

Excitation:	560
Emission:	600 or 605
Read Height:	**8.35 mm**

Bottom reading option

Plate	96 Well Falcon clear
PMT Gain:	Manual, 600 volts
Integration Time:	10 ms
Excitation:	560
Emission:	610