**BBTN-2019-0138 R1**

**Supplementary material**

**Experimental determination of lipid A-ligand interactions**

Lipid A, one of the most potent stimulators of the innate system, induces a wide spectrum of biological effects, which may be harmful for the hosts. Thus, theoretical, experimental and combined studies have been developed to analyze the interaction between the lipid A and different binding molecules. These approaches contribute to clarify the binding mechanism and make progress on advanced therapies for sepsis control. In this context, some authors focused their investigation on the kinetics and reaction mechanisms as they play an important role in the design and optimization of the analytical or treatment devices. Kinetic studies, whose main objective is the determination of the equilibrium constant from the kinetic parameters of both direct and reverse binding reactions, employ either surface plasmon resonance (SPR), micro calorimetry (ITC), or fluorescence resonance energy transfer (FRET).

***Surface Plasmon Resonance (SPR)***

Surface Plasmon Resonance is a mass-sensitive transductor procedure that monitors in real-time the association and dissociation events between a binding molecule immobilized on a surface and a partner injected on the surface. The sensor surface is composed of a thin gold film on a transparent material illuminated by polarized light as depicted in figure 3. Once the wavelength, angle and refractive indices are adjusted, resonance takes place between the light and the free electrons clouds (plasmon wave). Binding and dissociation between the immobilized compound and its partner change the refractive index and allows real-time tracking of the resonance conditions [139,140].

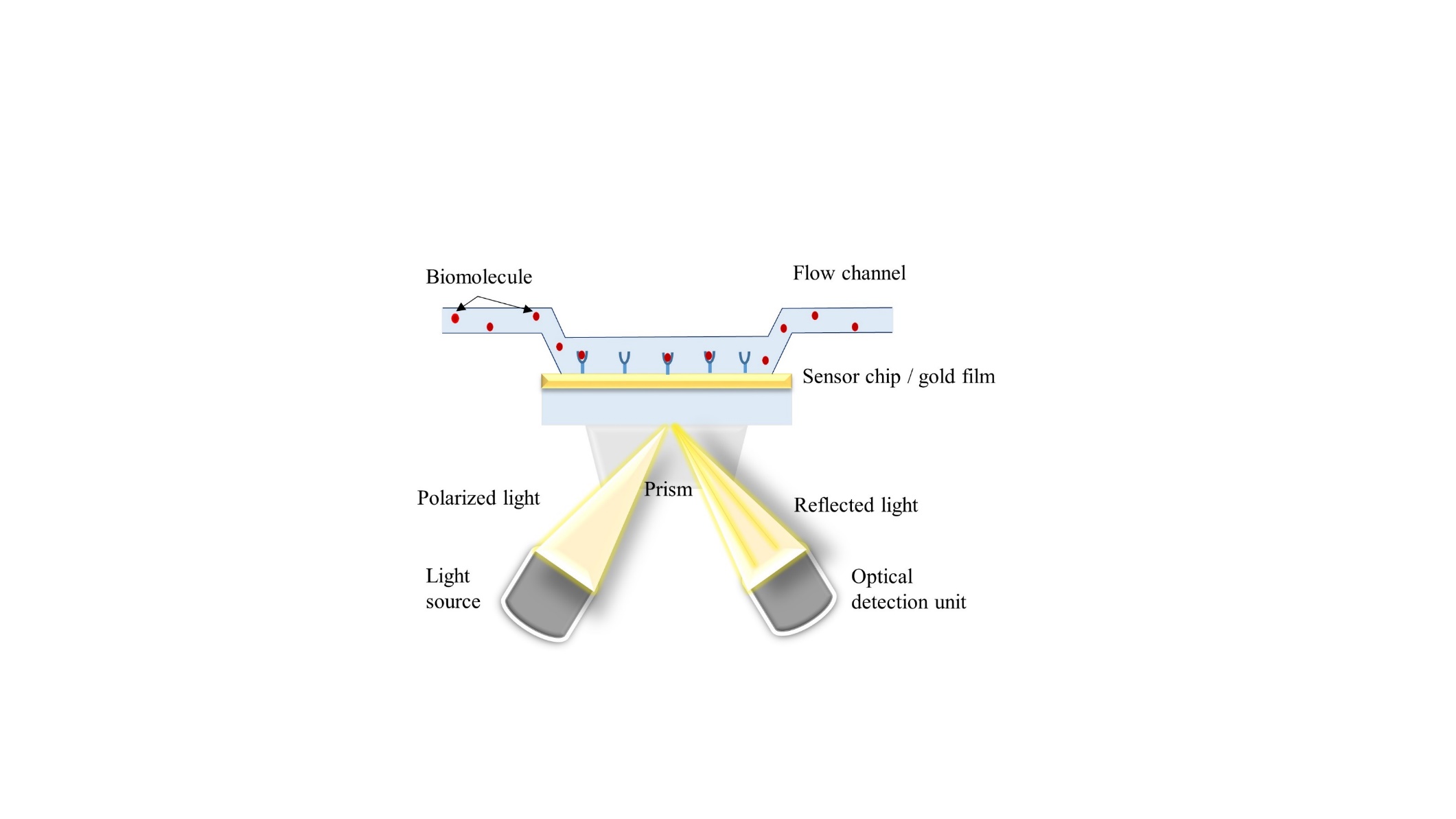


Figure 1. Surface Plasmon Resonance detection system

SPR screening offers detailed binding characteristics such as kinetic measurements, thermodynamic parameters and binding stoichiometry [141]. The kinetics measurement procedure implies the injection of different samples of analyte with already known concentrations on a surface with low ligand levels in order to favor kinetic conditions. The main instrument of SPR is the BiacoreTM biosensor, where k1 (M-1 s-1) is the rate of analyte-surface binding and the K-1 (s-1) is the analyte removed from the surface. As result, it is generated a primary sensogram that allows calculating interaction parameters like association (KA) or dissociation (KD) constants as well as the maximal binding capacity of the surface (Rmax) [142,143].

*3.1.2 Isothermal titration calorimetry.*

Isothermal titration calorimetry (ITC) is playing a key role in the exhaustive study of protein-ligand interactions. Measuring protocol involves two different cells: a cell with around 1mL of the reactant and a second cell for temperature reference. A syringe that also serves as stirrer injects the ligand to the sample cell. Initially, both cells are equilibrated at the desired temperature but, when the experiment is started, a constant power is supplied to the reference cell and a compensating one is also supplied to the other cell in order to equilibrate temperatures. Based on pre-set intervals, the ligand is added and the associated absorbed or released heat is compensated thanks to the power supplied to the initial cell as depicted in figure 4. The feedback-supplied power is directly proportional to the heat-flow (dQ/dt) [144,145] . As a result of this procedure, different panels show relevant information about the interaction as the association constant (KA), the enthalpy change (AH) and the stoichiometry (n) for the reaction.

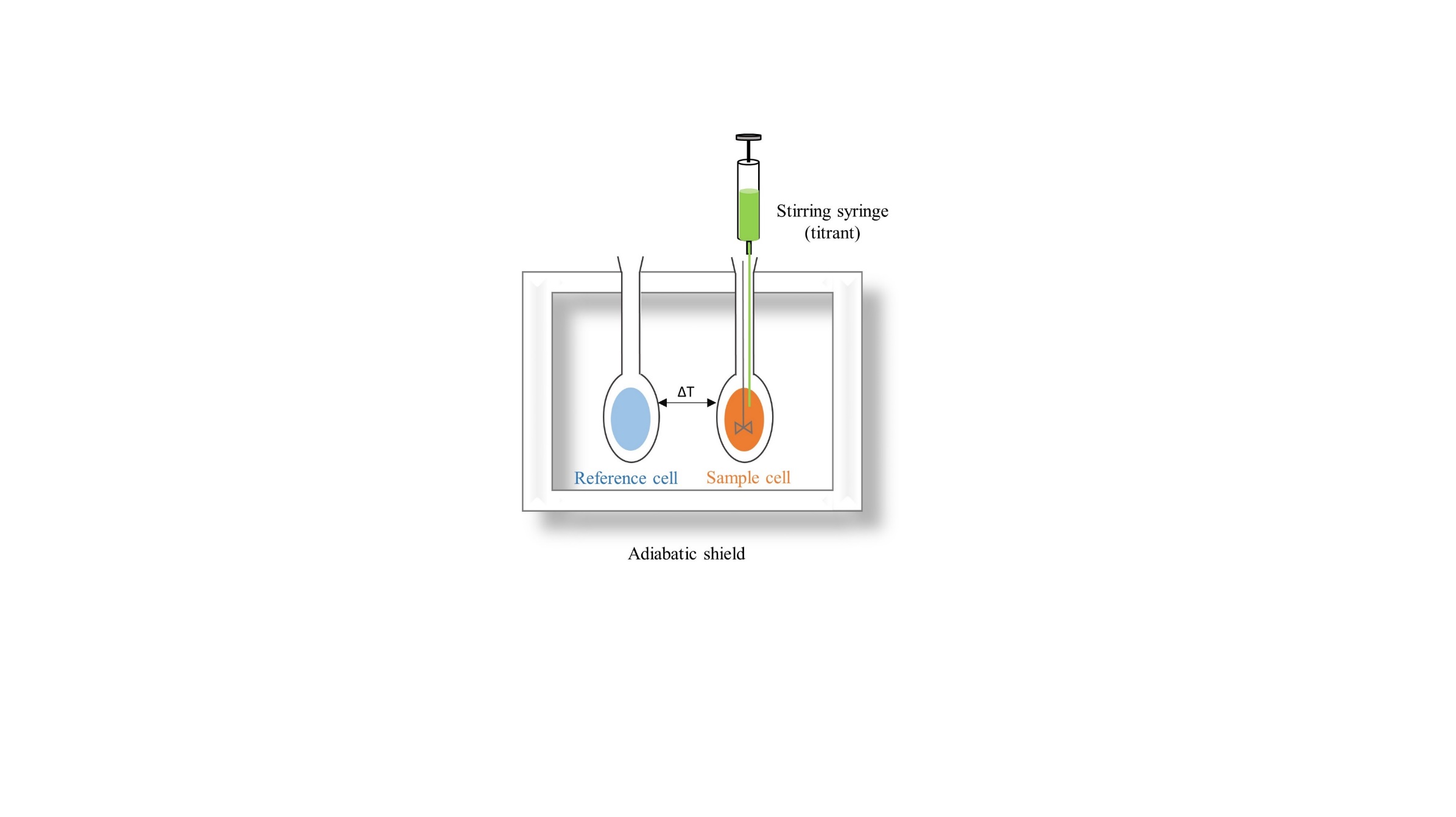


Figure 2. Isothermal Titration Calorimetry measurement system

*3.1.3 Fluorescence resonance energy transfer*

Fluorescence resonance energy transfer (FRET) is a useful technique based on the distance dependent transfer of energy between a donor fluorophore (D) and an acceptor fluorophore (A) whose absorption spectrum must partially overlap with the emission spectrum of D [146,147]. When both fluorophores are approached, the donor excited-stage energy is transferred through a dipole-dipole coupling. As a result of FRET, the fluorescence of the donor decreases and the fluorescence of A increases by means of the rate of energy transfer, KT, from D to A.

Fluorescence techniques allow determining kinetic rate constants under stopped-flow techniques of fast reactions. Stopped flow is a type of flow injection analysis where reactants are rapidly driven from syringes into a high efficiency mixer as shown in figure 3. To perform a run, two syringes are filled with the reagent, the content is expelled into the flow circuit, and a small volume of each reagent is displaced through the mixer observation cell. When the flow is stopped, the reaction initiated by mixing proceeds in the observation cell and the change in absorbance is monitored by the spectrophotometer following the absorbance change in the millisecond time range. The flow is stopped with the reactant stream in the flow cell photometric detector that usually is the fluorescence detector as it is more sensitive. The sensitivity of fluorescence detection is very useful to limit the amount of material needed by this technique. Kinetic determination is obtained by monitoring the concentration of the reactants over time using absorption or fluorescence spectroscopy [148,149].

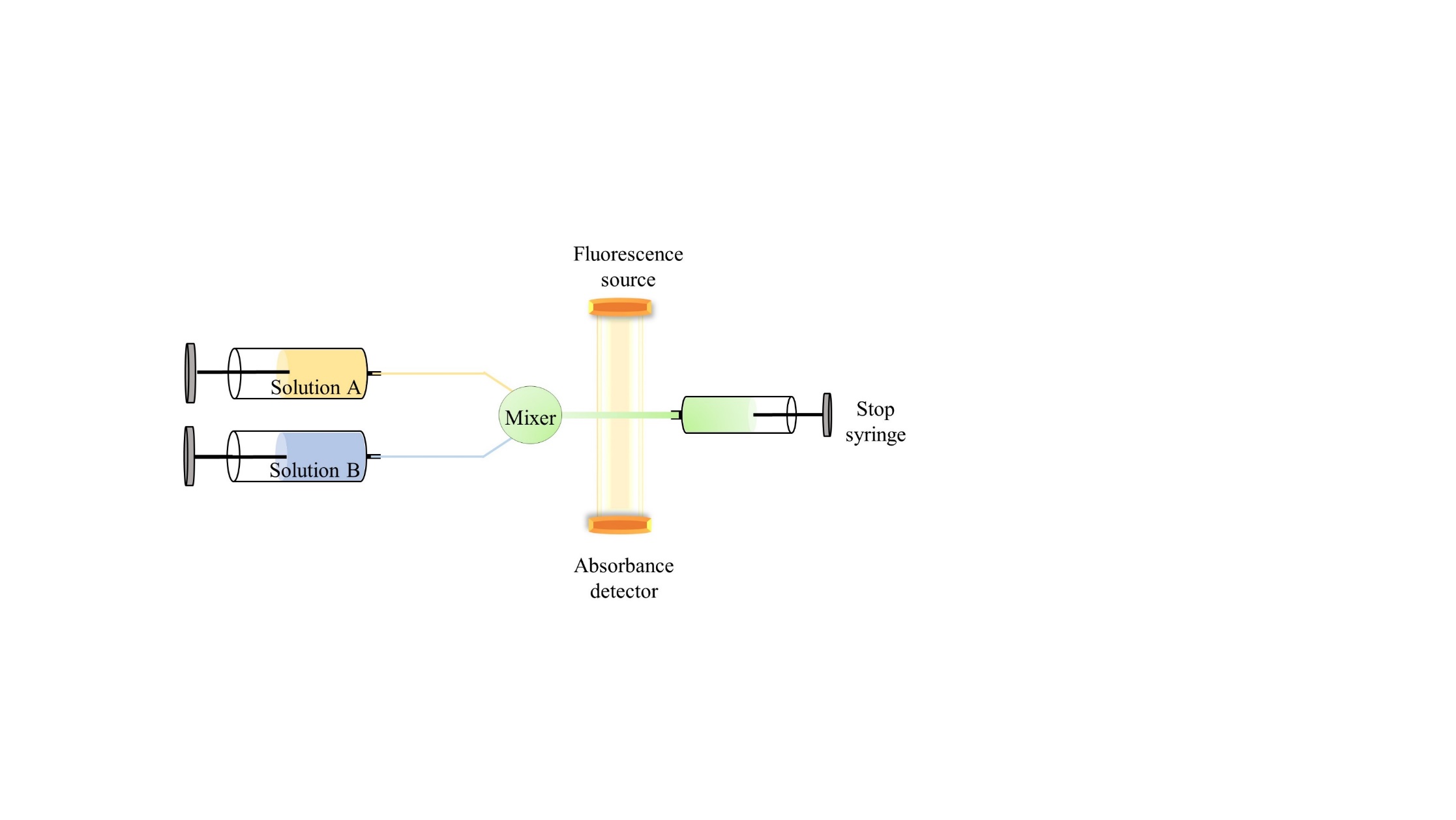


Figure 3. Stopped flow injection analysis system

**List of Abbreviations and Acronyms**

|  |  |
| --- | --- |
| BPI | Bactericidal permeability-increasing protein |
| CD14 | Cluster of differentiation 14 |
| CETP | Cholesterylester transfer protein |
| CXCR4 | Chemokine receptor 4 |
| Dansyl-PMB | N-dimethylaminonaphthalene-5-sulfonyl-PMB |
| FITC | Fluorescence isothiocyanate |
| FRET | Fluorescence resonance energy transfer |
| G (-) | Gram negative |
| GDF-5 | Growth differentiation factor 5 |
| H1, H2A, H2B, H3, H4 | Histones |
| HBP | Heparin binding protein |
| HDL | High-density lipoprotein |
| HNP | Human α-defensins |
| HSP70/ HSP90 | Heat shock proteins |
| ITC | Isothermal Titration calorimetry |
| KDO | [3-Deoxy-D-manno-octulosonic acid](https://en.wikipedia.org/wiki/3-Deoxy-D-manno-octulosonic_acid_kinase) |
| LAL | Limulus amebocyte lysate assay |
| LALF | *Limulus poplyphemus* |
| LBP | Lipid binding protein |
| LDL | Low-density protein |
| LPS | Lipopolysaccharides |
| MD2 | Myeloid differentiation protein |
| MoAbs | Monoclonal antibodies |
| NK cell | Natural killer cell |
| P2X7 | Nucleotide receptor |
| PEI | Polyethylenimine |
| PLTP | Phospholipid transfer protein |
| PMB | Polymyxin B |
| PMBN | Polymyxin B nonapeptide |
| RPT | Rabbit pyrogen test |
| rsCD14 | Recombinant soluble CD14 |
| sCD14 | Soluble CD14 |
| SLPI | Secretory protease inhibitor |
| SMAP-29, rCAP18, hCAP18 | Cathelicidins |
| SP-A, SP-D | Hydrophilic surfactant proteins |
| SP-C | Hydrophobic surfactant protein |
| SPR | Surface Plasmon Resonance |
| TALF | *Tachypleus tridentatus* |
| TLR | Toll like receptor |
| VLDL | Very-low density lipoproteins |