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Fetal Calf Serum (FCS / FBS) Protein Binding

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We use this protocol and it's working

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Abstract

In vitro pharmacological and toxicological evaluation of chemicals is performed using a wide variety of cell-based assay systems. One of the major factors influencing the in vitro responses of chemicals is the amount of unbound chemicals present at the target site. The concentrations of the unbound chemicals primarily depend on the protein levels and their binding affinities. Fetal calf/bovine serum (FCS / FBS) Protein binding assay is used in cell culture in cell biology research and in the pharmaceutical industry to support this analysis.

Assay to Determine Protein binding in fetal calf / bovine serum (FCS / FBS) capabilities for compounds and reference compound (Verapamil) - In 2% heat-inactivated FCS (30 min, 56°C) using HPLC-MS/MS. The task was performed by spiking test compounds at a concentration of 1 μ M into 2% FCS and dialyzing against buffer until equilibrium is achieved. Peak areas of the compound in both 2% FCS and buffer were determined to calculate the percentage of protein-bound compound.



Materials

Equipment:

- HPLC system with mass spectrometric detector API4000/API5000.
- Incubator Series II Water Jacketed CO2 incubator.
- Multi-channel automatic pipettes: 2-125 μ L, 1-30 μ L, 15-1250 μ L, E1-ClipTip.
- Reusable dialyzer HTD96b.
- Dialysis membrane strips (HTDialysis LLC, USA; cat. no. 1101).
- Disposable tips for Thermo automatic pipettes Scientific Cliptip.
- Polypropylene tubes in a 96-well rack, 0.75 ml.
- Silicone 96-well micromats with a round bottom, pre-perforated.

Reagents:

- 100 mM (1x) Potassium phosphate buffer (PPB), pH 7.4
- RPMI medium with 10% Fetal Bovine Serum (FBS / FCS)
- Compounds stock solutions: prepare 20 mM stocks of reference and test compounds in DMSO. Then, dissolve DMSO stock in acetonitrile (ACN) to 100 μ M, and mix by vortexing.
- Quenching solution with internal standards.
- Dissolve appropriate for LC-MS/MS analysis internal standards in ACN.

Safety warnings

- ! Always wear appropriate PPE for this protocol
Refer to Material Safety Data Sheets for additional safety and handling information.

Samples Preparation

6h 5m

1 Preparation of Phosphate-buffered saline (PBS):

- 1.1 PBS [1M] 10 millimolar (mM) (1X), pH 7.4. To prepare 10X ([1M] 100 millimolar (mM)), the buffer packet is first dissolved in 50 mL of MilliQ water. Filter the solution with a syringe filter, store at 4 °C no more than 4 weeks. Before the test, dilute the 10X solution to the working concentration of 1X ([1M] 10 millimolar (mM)), bring the pH to 7.4. [1M] 1 Mass Percent PBS store at 4 °C no more than 7 days.

2 Preparation of RPMI medium with 10% FBS / FCS:

- 2.1 To prepare 1 L of basic basicliquid medium from this item would require 10.4 g of the powder product plus the addition of 2 g of sodium bicarbonate. pH is regulated in a range of 7.3-7.6. Then media is filtered through PES filter-cup with 0.22 um pores. After the prepaton of basic media the heat-inactivated FBS is added in a final concentration of 10%. pH is regulated in a range of 7.3-7.6. Then media is filtered through PES filter-cup with 0.22 um pores. After the prepaton of basic media the heat-inactivated FBS is added in a final concentration of 10%.

3 ACN stock, [1M] 100 micromolar (μM) :

- 3.1 Add 995 μL of acetonitrile and 5 μL of [1M] 20 millimolar (mM) DMSO stock substance to a 1.4 mL microtube, mix by pipetting. Store no more than two weeks at 4 °C .



4 Preparation of dialysis membrane:

- 4.1 Before the test, cut off the required amount of the membrane strip, cut it lengthwise so that after soaking, they are divided into two single strips (Fig. 1). Place the membrane in

1h

MilliQ water or PBS for  01:00:00 . Divide the soaked membrane into two single strips.

Note

WARNING! Make sure only a single sheet of dialysis membrane is placed between Teflon bars.

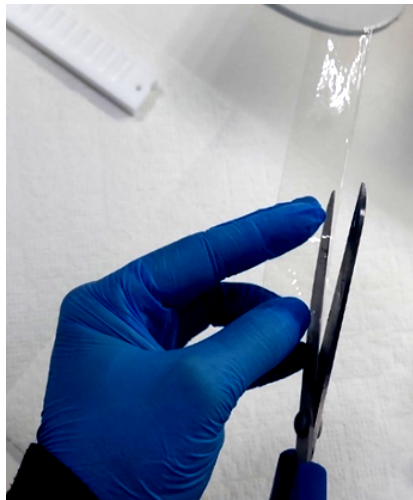


Fig. 1. Scheme of cutting the dialysis membrane before soaking.

5 Assembling the Teflon bars with dialysis membrane:

- 5.1 Lay the first Teflon bar flat on the bench (Fig. 2), insert the two stainless steel connecting rods so they are perpendicular to the Teflon bar. Place the membrane on the Teflon bar. Ensure that the membrane is approximately 2mm below the top edge of the bar and the lower membrane edge overlaps the bottom of all wells (Fig. 2a).

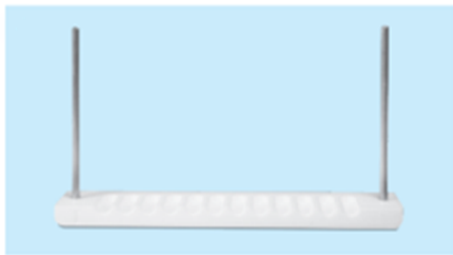
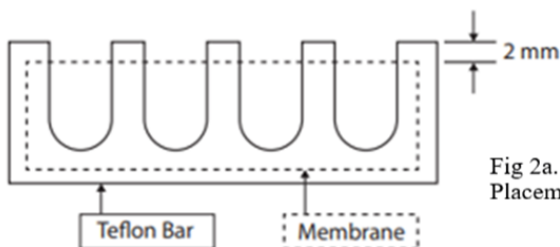


Fig.2 Teflon bar with steel connections

Fig 2a.
Placement of membrane on Teflon bar

6 Assembly of dialysis machine:

- 6.1 Take the machine and make sure that it is in the open position (Fig. 3). Insert the Teflon block into the machine and the pressure plate between the Teflon block and the pressure shaft as in (Fig. 3a). Tighten the assembled block, evenly rotating the cam levers with both hands (Fig. 3b).



Fig.3 Open position



Fig.3a Insert the Teflon block and pressure plate

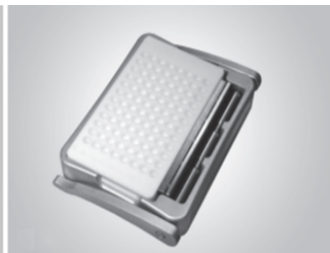


Fig.3b Tighten the assembled block

7 Carrying out the test:

- 7.1 Prepare [M] 1 micromolar (μM) model solutions of substances in RPMI medium with 10% FBS. Add $\text{3 } \mu\text{L}$ ACN substance in $\text{297 } \mu\text{L}$ of RPMI medium with 10% FBS



(Fig. 4). Mix by pipetting 4-5 times, pouring down the wall of the test tube.

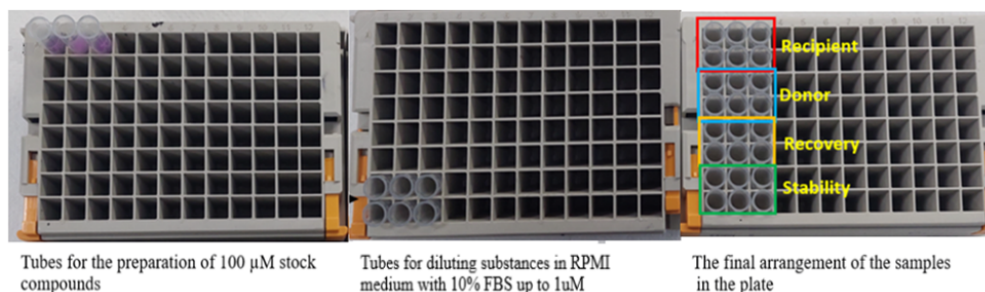


Fig. 4 Scheme of arrangement of microtubes in the plates

- 7.2 Add  125 μL of solutions of substances in RPMI medium with 10% FBS to the donor cells of the machine (Fig. 5). Add  50 μL each of model solution and PBS in the 'Stability' microtubes of the final 96-well plate. Quench the solution in microtubes 'Stability' with  400 μL IS solution, mix on a vortex and store at  4 $^{\circ}\text{C}$ until analysis.

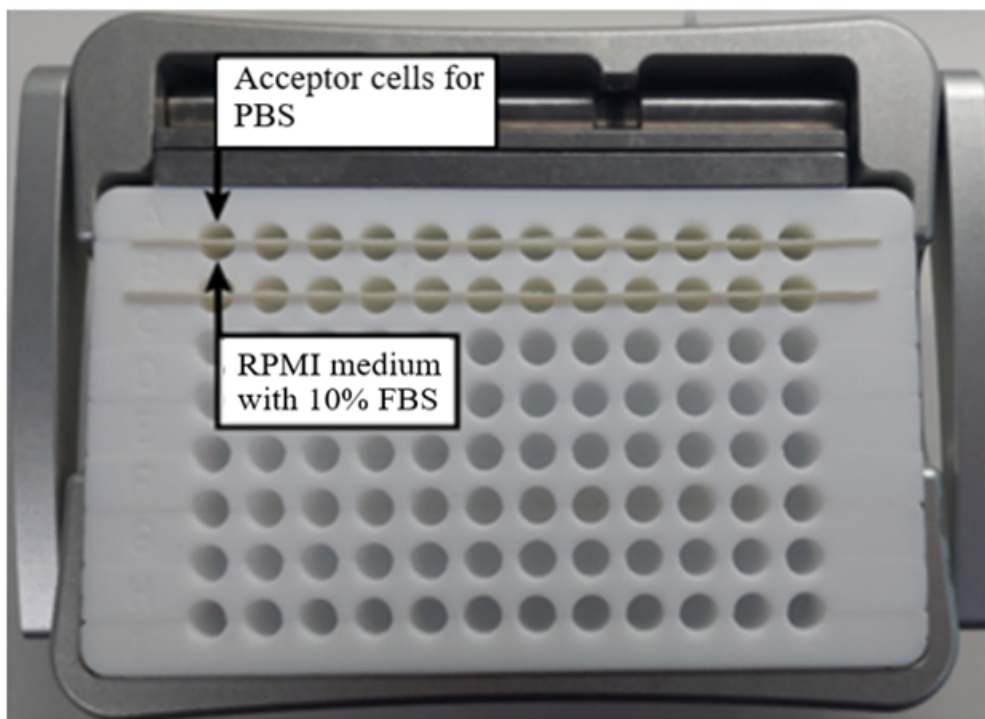


Fig 5. Schematic of a collective dialysis device



7.3 The dialysis device and model solutions in a CO₂ incubator and at 250 rpm and

37 °C for 05:00:00 .

5h



Note

WARNING! Most compounds reach equilibrium in less than 04:00:00 -

05:00:00 at 37 °C shaking at 250 rpm. For confidence, you can conduct the following kinetic experiment: compound spike into buffer and dialyze against buffer to evaluate the equilibrium time required prior to initiating any binding experiments.

8 After the incubation

8.1 To balance the matrix effect, add 50 µL blank RPMI medium with 10% FBS to the recipient tubes and vice versa, in the donor add recovery 50 µL of buffer. After that, transfer 50 µL of the acceptor solution to recipient tubes, 50 µL donor solution into donor tubes, and 50 µL of model solution to recovery tubes. All samples should be quenched immediately, by adding 400 µL of IS solution in recipient, donor, and recovery tubes. To check specificity and selectivity, should make a blank solution from the RPMI medium with 10% FBS.



8.2 And finally, close the test tubes with rubber caps, mix with a vortex and centrifuge for 6000 rpm, 00:05:00 . Supernatants are analyzed using the HPLC system coupled with tandem mass spectrometer.

5m



Data Analysis

9 The percentage of substances bound to FBS proteins recovery and stability are calculated according to the following equations.

$$\text{Protein binding, } f_b = \left(1 - \frac{\text{area ratio in buffer}}{\text{area ratio in plasma}}\right) \cdot 100\%$$

$$\text{Recovery} = \left(\frac{\text{area_ratio_in_buffer} + \text{area_ratio_in_RPMI medium with 10\% FBS}}{\text{area_ratio_in_recovery_sample}}\right) \cdot 100\%$$

$$\text{Stability} = \left(\frac{\text{area ratio in recovery sample}}{\text{area ratio in stability sample}}\right) \cdot 100\%$$

10 The percentage of unbound fraction f_u is calculated by the formula:

$$\text{Fraction unbound, } f_u = \left(\frac{\text{area ratio in buffer}}{\text{area_ratio_in_RPMI medium with 10\% FBS}}\right) \cdot 100\%$$

or

$$\text{Fraction unbound, } f_u = 100 - f_b$$