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**«ANTIBODY IMMOBILIZATION ON GLASS COVERSLIP»**

1. Centrifuge AB solution at 16kg (RCF) for 30min at +4°C to precipitate AB clusters. Using pipette take 80% of centrifuged solution from the tube. Pipette tip should always stay in the middle of solution volume and not contact surface or bottom of the solution
2. Using clean nitrile gloves manually wash and scrub glass coverslip (25x25mm) in 7x detergent until no visible spots, stains, dust etc.
3. Rinse the coverslip in mQ flow and place it in Petri’s dish filled with Isopropanol
4. Put Petri’s dish with coverslip on the horizontal shaker for 10 min (110RPM). Flip the coverslip in the Petri’s dish in the middle of washing on shaker
5. Repeat STEPs 2-3 in another Petri’s dish filled with Ethanol (95%)
6. Rinse the coverslip in mQ and dry it with pressured air or N2. Place it on the ceramic pedestal
7. Cut out PDMS cube ~25x25mm (Mask: 1mm width channel, 50µm height; chamber with 4 parallel flow channels)
8. Use only 2 central flow channels for next steps
9. Make diagonal punctures (~45˚ angle) starting at both ends of a flow channel and ending at the upper side of a PDMS cube (inlet and outlet)
10. Check if punctures are linear and straight and there are no ruptures of PDMS along punctures
11. Rinse PDMS cube first in ethanol and then in mQ water
12. Dry the PDMS cube until no visible drops of water (use pressured air or N2)
13. Activate PDMS cube and glass coverslip in Plasma cleaner at HIGH intensity, 400mTorr for 30sec (Follow «Plasma Cleaner protocol»)
14. Attach PDMS cube to the coverslip so that the lower side of the PDMS cube (open side of flow channels) connects to glass coverslip surface
15. Insert inlet and outlet tube for each channel into punctured holes
16. Outlet tubes should be connected to the 1ml Hamilton® glass syringes placed on a syringe pump
17. Place the open end of both inlet tubes inside the PBS aliquot
18. Perfuse PBS through the channels until it’s visible inside the outlet tube (~20-30 µl of buffer per channel). Syringe pump should perfuse at 20µl/min
19. Switch the PBS aliquot with 30µl AB aliquot (40ug/ml). AB solution should be preheated up to RT. Make sure there are no visible bubbles diluted in solution volume
20. Perfuse AB solution through the channels (~25-30 µl of solution per channel).
21. Stop the flow and leave the chamber to incubate for 1 hour at RT (if AB is fluorescent keep the chamber away from well-lit places)
22. Leave the open end of inlet tubes in AB aliquot so that air bubbles couldn’t get in the tube
23. Switch the AB aliquot with 1-1.5ml «washing» buffer (4%BSA) aliquot. Buffer should be preheated up to RT. Make sure there are no visible bubbles diluted in buffer volume
24. Use tweezers to move inlet tubes from one aliquot to another. Squeeze the tubes through the aliquot switching so that there are no air gaps inside the inlet tubes
25. Perfuse «washing» buffer through the channels for 4 min at 100µl/min
26. Stop the flow and incubate the chamber with 4% BSA buffer for 1 hour

Materials:

**Antibody**:
Mouse anti human CD31 (1mg/ml, Bio-Rad™, mono, #MCA1738T, USA).
Antibody is stored at -80°C. Stock aliquots – 2.5µl (333µg/ml, ~27% Glycerol, stored in PBS)
AB is diluted to 40ug/ml using dilution buffer

**Dilution buffer**:
PBS (pH 7.4): NaCl – 137 Mm; KCl – 2.7 mM; $Na\_{2} HPO\_{4} $– 10 mM; $KH\_{2} PO\_{4} $– 1.8 mM. (Stored at -20°C or at +4°C for near use).

**Washing buffer**:
Buffer A (pH 7.4) + 4% BSA: NaCl – 150 mM; KCl – 2.7 mM; MgCl2 – 1 mM; Na2HPO4 – 0.4 mM; HEPES – 20 mM; D-glucose – 5 mM; 4% Bovine Serum albumin (Stored at -20°C).

For PDMS cubes use «PDMS chambers preparation» protocol

**Inlet and outlet tubes** consist of blunt and bent needles (21g x2 0.8mm) and PEI tubes (0.6mm, 0.15mm). One end of the needle is inserted into PEI tube and fixed, other one is used to insert it into PDMS punctures. For inlet and outlet tubes preparation use «Reusable flow tubes preparation» protocol

**Syringes**:
Hamilton® GASTIGHT®, LTN (fixed needle) 1001LTN, volume 1 mL, needle size 22 ga (bevel tip), needle L 51 mm (2 in.) #20740-U. Syringe needle is connected to outlet tube

**Syringe pump**:
Parameters:
Flow rate = 100 or 20µl/min
Diameter = 4.8mm
Volume = 400µl
«Withdraw» mode