**Required solutions:**

Tris Buffer – dissolve 1 tablet Tris per 15 ml H20

50 mM glycine in Tris – 4 mg glycine in 1 ml Tris

1% Tween stock – 10 ul Tween in 1 ml H20

10% BSA stock – 10mg BSA in 1 ml H20

Blocking solution – 0.05% Tween and 0.1% BSA in Tris:

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  |  |  |  |  |  |  |
|  | Number of Ribbons | 1 | 2 | 3 | 4 | 5 |
| Blocking Solution | Final Volume | 1500uL | 2000uL | 3000uL | 4000ul | 5000uL |
| 10% BSA | 15uL | 20uL | 30uL | 40uL | 50uL |
| 1% Tween | 75uL | 100uL | 150uL | 200uL | 250uL |
| Tris Buffer | 1410uL | 1880uL | 2820uL | 3760uL | 4700uL |
| 1' Antibody Solution | Final Volume | 300uL | 600uL | 900uL | 1200uL | 1500uL |
| 1:100 | 3uL | 6uL | 9uL | 12uL | 15uL |
| 1:150 | 2uL | 4uL | 6uL | 8uL | 10uL |
| 1:200 | 1.5uL | 3uL | 4.5uL | 6uL | 7.5uL |
| 1:1000 | 0.3uL | 0.6uL | 0.9uL | 1.2uL | 1.5uL |
| Blocking Solution | subtract total antibody vol. from final vol. | | | | |
| 2' Antibody Solution | Final Volume | 300uL | 600uL | 900uL | 1200uL | 1500uL |
| Ab 1 (1:150) - 488 | 2uL | 4uL | 6uL | 8uL | 10uL |
| Ab 2 (1:150) - 594 | 2uL | 4uL | 6uL | 8uL | 10uL |
| Ab 3 (1:150) - 647 | 2uL | 4uL | 6uL | 8uL | 10uL |
| Blocking Solution | 294uL | 588uL | 882uL | 1178uL | 1470uL |

**Immunostaining:**

Ensure that coverslips are mounted in a metal frame, with a flow cell mounted on top of the ribbon. The flow cell should be well sealed to the glass.

If not already full of liquid, load the flow cell through the smaller well with 1% Photoflo in H20 solution.

After flow cell is loaded and free of any bubbles, follow these incubation steps:

5 min - 50mM glycine in Tris

5 min - Blocking solution

2 hrs to overnight – Primary antibody.

* To make primary antibody, follow the chart above for volumes of buffer and antibodies depending on number of ribbons and desired antibody concentration. Spin dow antibody solution at 13,00 rpm for 2 minutes before loading into flow cell.

3 x flush with 1X Tris

30 min – Secondary antibody

* To make secondary, wrap test tube in tin foil to block light, then follow the above chart. Alexa Fluor secondaries are used in a 1:150 concentration.

3 x flush with Tris

1x flush with distilled H20

Fill flow cell with 20% DAPI solution in H20

If done with final round of imaging, elute, rinse, and dry the ribbon for post-staining or longer-term storage.