Immunostaining spotted slides (no Autostainer used). When ‘bath’, do that while rocking. Each one-pad slide accommodates 1.5 ml of fluid, when using the Grace Bio-labs incubation chambers. Mindful how many slides to be stained, calculate the volumes needed (BSA, avidin, biotin, H2O2)

**Day 1. Duration: ~150 min**

Rehydration of slides:

 1 Take slides out of the 15 oC incubator, allow them to warm up to RT for 10 min

 2 Bath TBS + 0.1% Tween-20 for 10 min

Blocking steps:

3 protein block: TBS + 0.1% Tween + BSA (Sigma A7906) **5%**, for 60min

4 Add two drops of Avidin solution (VECTOR) per 10 ml TBS + 0.1% Tween *(no washing between protein block and Avidin)* for 10 min

5 Rinse TBS + 0.1% Tween

6 Bath TBS + 0.1% Tween for 10min

7 Add two drops of Biotin solution (VECTOR) per 10 ml TBS + 0.1% Tween for 10 min

8 Rinse TBS + 0.1% Tween

9 Bath TBS + 0.1% Tween for 10min

10 Add 0.3% H2O2 per slide for **15** min

11 Rinse TBS + 0.1% Tween

12 Bath TBS + 0.1% Tween for 10min

Primary antibody:

13 Add primary antibody diluted in TBS + 0.1% Tween + BSA **0.5%**. Cover the incubation chambers with the black lids, keep at 21°C overnight while gentle rocking.

**Day 2: Duration: ~5 hours**

Secondary antibody:

14 Aspirate primary antibodies from the incubation chambers

15 Rinse TBS + 0.1% Tween

16 Bath TBS + 0.1% Tween for 10 min

17 Bath TBS + 0.1% Tween for 10 min

18 Secondary antibody (HRP-conj.) 1/5,000 in TBS + 0.1% Tween + BSA **0.5%** for 120 min. Cover the incubation chambers with the black lids

Signal amplification:

19 Rinse TBS + 0.1% Tween

20 Bath TBS + 0.1% Tween for 10 min

21 Bath TBS + 0.1% Tween for 10 min

22 Bio-Rad Amplification Module for 15 min (2 parts of 2x “Amplification Diluent”, 1 part of 4x “Amplification reagent” and 1 part water)

23 Rinse TBS + 0.1% Tween

24 Bath TBS + 0.1% Tween for 10 min

25 Bath TBS + 0.1% Tween for 10 min

Fluorophore:

26 Streptavidine-IRDye800CW 1/2,000 in TBS + 0.1% Tween + BSA 0.5% for 60 min. Cover the incubation chambers with the black lids

27 Rinse TBS + 0.1% Tween

28 Bath TBS + 0.1% Tween for 10 min

29 Bath TBS + 0.1% Tween for 10 min

30 Dry arrays immediately by centrifugation for 5 min at 50g

31 Scan arrays using Innoscan 710-IR, using the 785 nm laser.