

ISH

Check availability of:

- Affymetrix reagents
- Probes
- 600ml 100% ethanol
- 400ml xylene
- 200ml Gill's hematoxylin
- DAPI store in dark at 4C)

Protocol – Day 1:

1. Set hybridization system to 60C
2. Set incubator to 40C and check that it is humidified
3. Label slides with a pencil
4. Bake slide for 30-60min in silver slide holder
5. Prepare Solutions:
 - a. 3l of 1X PBS (2.7l ddH₂O + 300ml 10X PBS) in 4l plastic container
 - i. prewarm 40ml 1x PBS & Probe Set Diluent QT to 40C
 - b. 500ml of 1X Pretreatment Solution (495ml ddH₂O + 5ml 100x Pretreatment Solution) in 1l glass beaker
 - i. turn on hot plate, cover beaker with foil, stick in thermometer and heat up to 90-95C
 - ii. KEEP CHECKING TEMPERATURE
 - c. 200ml of 10% NBF (178ml 1x PBS + 22ml 37% FA [flammable cabinet]) – white 200ml container - WORK IN FUME HOOD
 - d. 4l of Wash buffer (3l ddH₂O + 36ml Wash Comp 1 + 10ml Wash Comp2 + 954ml ddH₂O) – 4l plastic container
 - e. 200ml of Storage buffer (140ml ddH₂O + 60ml Wash Comp2) – white 200ml container with lid
 - f. 1l of 0.01% ammonium hydroxide (999.67ml ddH₂O + 330microl 30% ammonium hydroxide) – 1l glass bottle – WORK IN FUME HOOD
6. Take probe sets from freezer and place on ice

7. Pour 2x 200ml **xylene** into green 200ml container – WORK IN FUME HOOD
8. Pour 2x 200ml **100% ethanol** into white 200ml container
9. Transfer slides from silver metal slide holder to grey plastic slide holder
10. Incubate 5' in xylene (1) – agitate frequently
11. Incubate 5' in xylene (2) – agitate frequently
12. Incubate 5' in ethanol (1) – agitate frequently
13. Incubate 5' in ethanol (2) – agitate frequently
14. Place slides on paper towel and air dry for 5'
15. Draw hydrophobic barrier and air dry for **20-30'**
16. Load slides in grey plastic slide holder and submerge into heated **1x Pre-Treatment solution** – **10'** for kidney (5' for lung) – cover glass beaker with foil
17. Pour 2x 200ml of **ddH₂O** in 200ml white containers
18. Pour 1x 200ml **PBS** in 200ml white container
19. Prepare Working Protease Solution (396microl warm 1x PBS + 4microl Protease QF / slide) – make 1 extra
20. Incubate 1' in ddH₂O (1) – agitate frequently
21. Incubate 1' in ddH₂O (2) – agitate frequently
22. Transfer slides into PBS
23. Flick off + wipe backside
24. Transfer slides onto metal rack
25. Add 400microl of **Working Protease Solution** – incubate **15'** for kidney (10' for lung) at 40C
26. Pour 2x 200ml 1x **PBS** in 200ml white container
27. Pour 200ml **10% NBF** (FA) in 200ml white container – WORK IN FUME HOOD
28. Incubate 1' in PBS (1) – agitate *gently*
29. Incubate 1' in PBS (2) – agitate *gently*
30. Incubate 5' in 10% NBF – WORK IN FUME HOOD
31. Pour 2x 200ml 1x **PBS** in 200ml white container
32. Prepare Working Probe Set Solution (390microl warm Probe Set Diluent QT + 10microl ViewRNA probe set / slide) – make 1 extra
33. Incubate 1' in PBS (1) – agitate frequently

34. Incubate 1' in PBS (2) – agitate frequently
35. Flick off + wipe backside
36. Add 400microl of **Working Probe Set Solution** – ONLY PROBE SET DILUENT
ON NEGATIVE CONTROL - incubate for **2hrs** at 40C
37. Pour 3x 200ml **wash buffer** in 200ml white container
38. Incubate 2' in wash buffer (1) – agitate frequently
39. Incubate 2' in wash buffer (2) – agitate frequently
40. Incubate 2' in wash buffer (3) – agitate frequently
41. Pour 200ml **storage buffer** in 200ml white container + lid
42. Store at RT **up to 24hrs**

Protocol – Day 2:

43. Prewarm PreAmplifier Mix QT, Amplifier Mix QT and Label Probe Diluent QF at 40C
44. Bring AP Enhancer Solution, Naphtol Buffer and Fast Red Tablets to RT
45. Put Label Probe 1-AP on ice
46. Pour 2x 200ml **wash buffer** in 200ml white containers
47. Incubate 2' in wash buffer (1) – agitate frequently
48. Incubate 2' in wash buffer (2) – agitate frequently
49. Flick off + wipe backside
50. Add 400microl of warm **PreAmplifier Mix QT** – incubate for **25'** at 40C
51. Pour 3x 200ml **wash buffer** in 200ml white containers
52. Incubate 2' in wash buffer (1) – agitate frequently
53. Incubate 2' in wash buffer (2) – agitate frequently
54. Incubate 2' in wash buffer (3) – agitate frequently
55. Flick off + wipe backside
56. Add 400microl of warm **Amplifier Mix QT** – incubate for **15'** at 40C
57. Pour 3x 200ml **wash buffer** in 200ml white containers
58. Prepare Label Probe 1-AP solution (399.6microl warm Label Probe Diluent QF + 0.4microl Label Probe 1-AP / slide) – make 1 extra
59. Incubate 2' in wash buffer (1) – agitate frequently

60. Incubate 2' in wash buffer (2) – agitate frequently
61. Incubate 2' in wash buffer (3) – agitate frequently
62. Flick off + wipe backside
63. Add 400microl of Label Probe 1-AP solution – incubate for 15' at 40C
64. Pour 3x 200ml wash buffer in 200ml white containers
65. Incubate 3' in wash buffer (1) – agitate frequently
66. Incubate 3' in wash buffer (2) – agitate frequently
67. Incubate 3' in wash buffer (3) – agitate frequently
68. Flick off + wipe backside
69. Add 400microl of AP Enhancer – incubate 5' at RT
70. Prepare Fast Red Substrate (5ml Naphtol buffer + 1 Fast Red tablet -> vortex + keep dark)
71. Flick off 2x + wipe backside
72. Add 400microl of Fast Red Substrate – incubate for 30' at 40C
73. Pour 200ml of 1x PBS in 200ml white container
74. Pour 200ml hematoxylin into white plastic container – WORK IN FUME HOOD
75. Pour 3x 200ml of ddH2O in 200ml white containers
76. Transfer slides into grey slide holder
77. Incubate 1' in PBS – agitate frequently
78. Incubate for 5-10sec in hematoxylin
79. Incubate 1' in ddH2O (1) – agitate frequently
80. Incubate 1' in ddH2O (2) – agitate frequently
81. Incubate 1' in ddH2O (3) – agitate frequently
82. Pour 200ml 0.01% ammonium hydroxide in 200ml white container – WORK IN FUME HOOD
83. Pour 200ml ddH2O in 200ml white container
84. Incubate 10sec in 0.01% ammonium hydroxide
85. Incubate 1' in ddH2O – agitate frequently
86. Flick off + wipe backside
87. Add 400microl of DAPI- incubate 5' at RT
88. Pour 200ml of ddH2O in 200ml white container

89. Incubate 1' in ddH₂O – agitate frequently

90. Air-dry slides in the dark ~ 20min

91. Mount slides with Fluoromount Aqueous (clear bottle) – WORK IN FUME HOOD

92. Store in the dark at 4C