PÉCSI TUDOMÁNYEGYETEM
ÁLTALÁNOS ORVOSTUDOMÁNYI KAR

WORLD-CLASS RESEARCH CENTER
PAVLOV CENTER
INTEGRATIVE PHYSIOLOGY
TO MEDICINE, HIGH-TECH HEALTHCARE
AND TECHNOLOGIES OF STRESS RESISTANCE

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# THE MANUAL

# ON THE RNAscope

A Guide for “Pavlov Center—Integrative Physiology to Medicine, High-tech Healthcare and Technologies of Stress Resistance” (supported by Ministry of Science and High Education of Russian Federation, grant № 075-15-2022-303 from 21.04.2022).

## SUMMARY

The RNAscope™ *in situ* hybridization (ISH) technology provides a powerful method to detect gene expression within the spatial and morphological tissue context. The proprietary “double Z” probe design in combination with the advanced signal amplification enables highly specific and sensitive detection of target RNA with single molecule and single cell resolution.

This book is a guide for researchers on the use of RNAscope in FFPE samples, cells or vibratom slices. It contains basic requirements and optimized protocols for establishing techniques from scratch, based on:

-USM-323100 Multiplex Fluorescent v2 User Manual_Support_04032017-1

-RNAscope, A Novel in Situ RNA Analysis Platform for Formalin-Fixed, Paraffin-Embedded Tissues, Wang et al. J Mol Diagn. 2012 Jan; 14(1): 22–29. doi: 10.1016/j.jmoldx.2011.08.002

## *Abbreviations used:*

Cy3: Cyanine 3
Cy5: Cyanine 5
DAPI: 4’ ,6-diamidino-2-phenylindole
FFPE: Formalin-fixed paraffin-embedded
HRP: Horseradish peroxidase
MQ: Milli Q water
PBS: Phosphate-buffered saline
PFA: Paraformaldehyde
TSA: Tyrmide signal amplification

## *Necessary reagents and equipment:*

RNAscope® Multiplex Fluorescent Reagent Kit v2 (Cat. No. 323100)
RNAscope® Multiplex TSA Buffer (Cat. No. 322809)
50x Wash Buffer Reagents (Cat. No. 310091)
TSA Plus fluorephores from Akoya or Invitrogen
RNAscope probes
Superfrost Ultra Plus/Gold slides
HybEZ oven (+tray, frame, paper, lid), black moist chambers
Prolong Gold/glass, Cover glass (24x50 mm)
Sterile MQ, sterile PBS
Repellent PAP Pen (Immedge from Vector or Superpap from Invitrogen)
Epp. tubes, Pipettes and pipette tips (filtered, sterile), plates
Kimtech tissue paper
Vortex, spin
Confocal microscopy

# Part 1: RNAscope on FFPE samples

**Important, general comments**

- Follow promptly the protocol in the indicated order
- Never let samples dry out
- Use fresh MQ water, do not need to autoclave water
- 1 or 2 day procedures are possible
- Pretreatment and assay according to RNAscope® Multiplex Fluorescent Reagent Kit v2 User Manual

## I. FFPE SAMPLE PREPARATION

- Whole animal perfusion-postfixation
  1. Work under chemical hood
  2. 1x PBS: must be cooled down, Filtered 4% PFA (or 10% NBF)
  3. For brain dissection: do not tape animals!
  4. Postfixation: ideally for 24 hr at 4C
  5. Wash organs in 1x PBS
  6. Store at 4C for few days in 1x PBS or longer in 1x PBS-0.01% Na-azide

- Sectioning
  1. Embed sample in paraffin using standard procedure, store blocks up to 6 months at 4C
  2. Spray water bath with 70% EtOH, fill with new MQ water
  3. Warm up water bath to 48-50C for brain, 46-48C for trigeminal ganglia
  4. Trim blocks into 5 um sections
  5. Place paraffin 1-3 ribbons onto Superfrost Ultra Plus slides
  6. Air-dry slide for few hours at RT
  7. Store sections at 4 C up to 6 months

## II. FFPE SAMPLE PRETREATMENT (DAY 1)

- Baking slides:
  1. Bake slide for 1 hr at 60C
  2. Leave sections at RT (optional stopping point, can be done on the previous afternoon)

- Preparations:
  1. Set oven/HybEZ oven to 40 C and 60 C
  2. **Fill and preheat** in the steamer:
     - 1 blue slide holder with 200 ml MQ,
     - 1 blue slide holder with 200 ml 1x Target Retrieval

- Deparaffinization under chemical hood at RT!

  (use solvents max. 3 times)

  1. **xylene 1.** for 5 min.
  2. **Agitate the slides** by occasionally lifting the slide rack up and down in the dish.
  3. Remove access of xylene on papertowel, **do not dry out samples!**

  4. **xylene 2.** for 5 min.
  5. **Agitate the slides** by occasionally lifting the slide rack up and down in the dish.
  6. Remove access of xylene on papertowel, **do not dry out samples!**

  7. **100% EtOH 1.** for 2 min.
  8. **Agitate the slides** by occasionally lifting the slide rack up and down in the dish.
  9. Remove access of EtOH on papertowel, **do not dry out samples!**

  10. **100% EtOH 2.** for 2 min.
  11. **Agitate the slides** by occasionally lifting the slide rack up and down in the dish.
  12. Remove access of EtOH on papertowel, **do not dry out samples!**
  13. Dry slides horizontally for 5 min at 58-60C

- $H_2O_2$-treatment
  1. Cover the whole section with 1-3 drop of $H_2O_2$
  2. Incubate promptly for 10 min at RT
  3. Rinse in MQ twice

- Target retrieval using **steamer**
  1. Submerge slides into hot MQ water for 10 sec
  2. Submerged slides into hot 1x Target Retrieval
  3. Steam for 15 min for most tissues, otherwise see at: Multiplex Fluorescent assay protocol page 36
  4. **Remove slides from the steamer as quickly as possible**
  5. Rinse in MQ for 15 sec
  6. Transfer slides to 100% EtOH for 3 min at RT
  7. Dry slides horizontally for 5 min at 58-60C

- Target retrieval using **Protease Plus in HybEZ tray or black moist chamber**
  1. Make sure that Immedge PAP-PEN do not leak! Try on the glass bench
  2. Draw a circle around the section
  3. Air-dry for 1 min
  4. Optional stopping point! Slides can stay at RT
  5. Moist paper of HybEZ tray/black moist chambers
  6. Add 1-3 drops Protease Plus/sample
  7. Incubate for 30 min at 40C (see further at Multiplex Fluorescent assay protocol, page 36)
  8. Meanwhile, prepare probe dilutions, see below

9. Rinse twice in MQ
10. Keep slides in MQ until probe hybridization

## III. PROBE HYBRIDIZATION (DAY 1)

**Important:**

- **Never let samples dry out!**
- **Make sure that moist chamber in always wet**
- **Remove bubbles**
- **During wash steps: rock slides gently with lid on top**

Probe dilutions:

- Warm up probe stocks to 40C (10-20 min), vortex-spin
- Dilute probes:
  - C1-probes: ready to use
  - C2, C3 probes: dilute to 1:50 in Probe diluent (PD) or in C1-probe
  - Diluted can be prepared on the previous (can be store at 4 C up to 6 months)
  - Right before usage, always vortex-spin/resuspend diluted probes
- Necessary ctrls:
  - Human, mouse, rat 3-plex pos ctrl
  - 3-plex neg. ctrl
  - singleplex ctrl
  - Pos. tissue ctrl
- Volumes: 50-250 ul depending on the sample size (from mouse coronal tissue to e.g. bigger human sample)

1. **Probe hybridization: 2 hr at 40 C in HybEZ oven**
2. Warm up 50x Wash buffer to 40 C (10 min), prepare 1X Wash in fresh MQ
   1x Wash can be stored at RT up to 1 month
   *For 2 L 1x Wash buffer: 40 ml prewarmed-50x Wash + MQ up to 2 L*
3. 2x 2 min 1x Wash at RT
4. Optinal stopping point: leave slides in 5x SSC-ben at RT in HyBEZ tray or in blue slide holder
   *For 300 ml 5x SSC: 75 ml 20x SSC + 225 ml MQ*

**20x SSC pH 7.4**

- 175 g NaCl
- 88.2 g Na-citrate
- 1L ddH2O
- adjust pH, autoclave, store at RT

## IV. SIGNAL AMPLIFICATION, CHANNEL DEVELOPMENT (DAY 2)

**Important:**

- **Never let samples dry out!**
- **Make sure that moist chamber in always wet**
- **Remove bubbles**
- **During wash steps: rock slides gently with lid on top**

- **Place AMP1, 2, 3 and HRPC1, 2, 3, HRP-blocker at RT (if slides are incubated in the HyBEZ oven. If not: prewarm the upcoming reagent to 40C)**
- **2. wash buffer can be reused as the following 1. wash buffer**
- **Make sure that the hydrophobe circle is visible before each dropping**
- **The protocol is optimized for:**
  **Channel 1: fluorescein**
  **Channel 2: Cy3**
  **Channel 3: Cy5**
  **but channels and fluorophores can be mixed.**

- <u>Signal amplification</u>

1. Warm up AMP1 at 40C and place AMP2, 3, HRPC1, 2, 3, HRP blocker at RT
2. 1x rinse in 1x Wash
3. Remove slides from tray/place slide back into black moist chamber
4. Add 1-4 drops of **AMP1**/sample
5. **30 min** at 40C
6. 2 min: 1x Wash at RT
7. 2 min: 1x Wash at RT

8. Remove slides from tray/place slide into black moist chamber
9. Add 1-4 drops of **AMP2**/sample
10. **30 min** at 40C
11. 2 min: 1x Wash at RT
12. 2 min: 1x Wash at RT

13. Remove slides from tray/place slide into black moist chamber
14. Add 1-4 drops of **AMP3**/sample
15. **15 min** at 40C
16. 2 min: 1x Wash at RT
17. 2 min: 1x Wash at RT

- <u>Channel 1 development</u>
  **Cover slides during washing steps, fluorophores are light-sensitive!**

1. Remove slides from tray/place slide into black moist chamber

2. Add 1-4 drops of **HRP-C1**/sample
3. **15 min** at 40C
4. Meanwhile, dilute fluorophores 1:750-1:3000 in TSA buffer, vortex and keep them at RT in dark
5. 2 min: 1x Wash at RT
6. 2 min: 1x Wash at RT
7. Remove slides from tray/place slide into black moist chamber
8. Add 50-250 ul of vortexed RT-**TSA Plus Fluorescein**/sample
9. **30 min** at 40C
10. 2 min: 1x Wash at RT
11. 2 min: 1x Wash at RT
12. Remove slides from tray/place slide into black moist chamber
13. Add 1-4 drops of RT-**HRP blocker**/sample
14. **15 min** at 40C
15. 2 min: 1x Wash at RT
16. 2 min: 1x Wash at RT

- Channel 2 development

1. Remove slides from tray/place slide into black moist chamber
2. Add 1-4 drops of **HRP-C2**/sample
3. **15 min** at 40C
4. 2 min: 1x Wash at RT
5. 2 min: 1x Wash at RT
6. Remove slides from tray/place slide into black moist chamber
7. Add 50-250 ul of vortexed RT-**TSA Plus Cy3**/sample
8. **30 min** at 40C
9. 2 min: 1x Wash at RT
10. 2 min: 1x Wash at RT
11. Remove slides from tray/place slide into black moist chamber
12. Add 1-3 drops of RT-**HRP blocker**/sample
13. **15 min** at 40C
14. 2 min: 1x Wash at RT
15. 2 min: 1x Wash at RT

- Channel 3 development

1. Remove slides from tray/place slide into black moist chamber
2. Add 1-3 drops of **HRP-C3**/sample
3. **15 min** at 40C
4. 2 min: 1x Wash at RT
5. 2 min: 1x Wash at RT

6. Remove slides from tray/place slide into black moist chamber
7. Add 50-250 ul of vortexed RT-**TSA Plus Cy5**/sample
8. **30 min** at 40C
9. 2 min: 1x Wash at RT
10. 2 min: 1x Wash at RT
11. Remove slides from tray/place slide into black moist chamber
12. Add 1-3 drops of RT-**HRP blocker**/sample
13. **15 min** at 40C
14. 2 min: 1x Wash at RT
15. 2 min: 1x Wash at RT

## V. MOUNTING AND STORAGE

1. 1-3 drops DAPI for 30 sec
2. 1x Wash at RT
3. 1x PBS rinse
4. Mount as soon as possible after DAPI staining
5. Remove big drops of 1xPBS but do not air-dry slides
6. Add 1 drop Prolong Glass antifade mountant onto sample
7. Dry slides horizontally in dry black moist chamber at 4C
8. Image preferable in 2-3 weeks
9. Store at -20C

## Part 2: RNAscope on cells (coverslipped, cytospinned)

**Important, general comments**

- Follow promptly the protocol in the indicated order
- Never let cells dry out
- Use fresh MQ water, do not need to autoclave water
- 1 or 2 day procedures are possible
- Sample type:
  - cytospinned cells or cells cultured (**50 ul/sample**), use Immedge Pap-Pen (draw circle on dry slides but do not let sample to dry out!!) **(NO bake!!!)**
  - glass coverslips in 24 well plate (**300 ul-5 droplets/ sample**)

## VI. FIXATION/STORAGE

1. Cytospin cells, air dry for appr. 30 min. or remove cell culture media from 24 well plate
2. Fixation in 10% NBF (Leica or Sigma) for 30 min at RT
3. 3x 1xPBS rinse/wash
4. Store at 4 C up to 2 months or:
5. 50 % EtOH for 5 min
6. 70 % EtOH for 5 min
7. 100 % EtOH for 5 min
8. 100 % EtOH for 10 min
9. Store at -20 C up to 6 months in 100 % EtOH

## VII. PRETREAMENT (DAY 1)

- Rehydration, if needed:
  1. 70% EtOH for 2 min
  2. 50% EtOH for 2 min
  3. 1x PBS rinse
  4. 1x PBS for 10 min

- Pretreatment
  1. 1/10 diluted Prot III. for 10 min at RT (dilution in 1x PBS)
  2. 3 x PBS rinse

## VIII. PROBE HYBRIDIZATION (DAY 1)

**Important:**

- **Never let samples dry out!**
- **Make sure that moist chamber in always wet**
- **Remove bubbles**
- **During wash steps: rock slides gently with lid on top**

- <u>Probe dilutions:</u>
  - Warm up probe stocks to 40C (10-20 min), vortex-spin
  - Dilute probes:
    - C1-probes: ready to use
    - C2, C3 probes: dilute to 1:50 in Probe diluent (PD) or in C1-probe
    - Diluted can be prepared on the previous (can be store at 4 C up to 6 months)
    - Right before usage, always vortex-spin/resuspend diluted probes
  - Necessary ctrls:
    - Human, mouse, rat 3-plex pos ctrl, 3-plex neg. Ctrl, singleplex ctrl, Pos. tissue ctrl

5. **Probe hybridization: 2 hr at 40 C**
6. Warm up 50x Wash buffer to 40 C (10 min), prepare 1X Wash in fresh MQ
   1x Wash can be stored at RT up to 1 month
   *For 0.5 L 1x Wash buffer: 10 ml prewarmed-50x Wash + MQ up to 0.5 L*
7. 2x 2 min 1x Wash at RT
8. Optinal stopping point: leave coverslips or slides in 5x SSC-ben at RT in HyBEZ tray or in the 24 well plate

## IX. SIGNAL AMPLIFICATION, CHANNEL DEVELOPMENT (DAY 2)

**Important:**

- Place AMP1, 2, 3 and HRPC1, 2, 3, HRP-blocker at RT (if slides are incubated in the HybEZ oven. If not: prewarm the upcoming reagent to 40C)
- 2. wash buffer can be reused as the following 1. wash buffer
- Make sure that the hydrophobe circle is visible before each dropping
- **It is not necessary to perform all the 3 channel development, but AMP1, AMP2 and AMP3 must be done!!!**
- **no AMP4 is!!! Only AMP1-2-3 even with channel 4 development**
- The protocol is optimized for:
  Channel 1: fluorescein

Channel 2: Cy3
Channel 3: Cy5
Channel 4: A594
**but channels and fluorophores can be mixed.**

- Signal amplification

18. Warm up AMP1 at 40C and place AMP2, 3, HRPC1, 2, 3, HRP blocker at RT
19. 1x rinse in 1x Wash
20. Add 1-5 droplets of **AMP1**/sample
21. **30 min** at 40C
22. 2 min: 1x Wash at RT
23. 2 min: 1x Wash at RT
24. Place slides back to moist chamber, check circles!
25. Add 1-5 droplets of **AMP2**/sample
26. **30 min** at 40C
27. 2 min: 1x Wash at RT
28. 2 min: 1x Wash at RT
29. Place slides back to moist chamber, check circles!
30. Add 1-5 droplets of **AMP3**/sample
31. **15 min** at 40C
32. 2 min: 1x Wash at RT
33. 2 min: 1x Wash at RT

- Channel 1 development

**Cover slides during washing steps, fluorophores are light-sensitive!**

17. Place slides back to moist chamber, check circles!
18. Add 1-5 droplets of **HRP-C1**/sample
19. **15 min** at 40C
20. Meanwhile, dilute fluorophores 1:750-1:3000 in TSA buffer, vortex and keep them at RT in dark
21. 2 min: 1x Wash at RT
22. 2 min: 1x Wash at RT
23. Place slides back to moist chamber, check circles!
24. Add 50-300 ul of vortexed RT-**TSA Plus Fluorescein**/sample

25. **30 min** at 40C
26. 2 min: 1x Wash at RT
27. 2 min: 1x Wash at RT
28. Place slides back to moist chamber, check circles!
29. Add 1-5 droplets of RT-**HRP blocker**/sample
30. **15 min** at 40C
31. 2 min: 1x Wash at RT
32. 2 min: 1x Wash at RT

- Channel 2 development

1. Place slides back to moist chamber, check circles!
2. Add 1-5 droplets of **HRP-C2**/sample
3. **15 min** at 40C
4. Meanwhile, dilute fluorophores 1:750-1:3000 in TSA buffer, vortex and keep them at RT in dark
5. 2 min: 1x Wash at RT
6. 2 min: 1x Wash at RT
7. Place slides back to moist chamber, check circles!
8. Add 50-300 ul of vortexed RT-**TSA Plus Cy3**/sample
9. **30 min** at 40C
10. 2 min: 1x Wash at RT
11. 2 min: 1x Wash at RT
12. Place slides back to moist chamber, check circles!
13. Add 1-5 droplets of RT-**HRP blocker**/sample
14. **15 min** at 40C
15. 2 min: 1x Wash at RT
16. 2 min: 1x Wash at RT

- Channel 3 development

1. Place slides back to moist chamber, check circles!
2. Add 1-5 droplets of **HRP-C3**/sample
3. **15 min** at 40C
4. Meanwhile, dilute fluorophores 1:750-1:3000 in TSA buffer, vortex and keep them at RT in dark
5. 2 min: 1x Wash at RT
6. 2 min: 1x Wash at RT

7. Place slides back to moist chamber, check circles!
8. Add 50-300 ul of vortexed RT-**TSA Plus Cy5**/sample
9. **30 min** at 40C
10. 2 min: 1x Wash at RT
11. 2 min: 1x Wash at RT
12. Place slides back to moist chamber, check circles!
13. Add 1-5 droplets of RT-**HRP blocker**/sample
14. **15 min** at 40C
15. 2 min: 1x Wash at RT
16. 2 min: 1x Wash at RT

**Continue with mounting or channel 4 development**

- Channel 4 development

Based on: 323120-TN 4-Plex Ancillary Kit for Manual multiplex_incOpal780-1
**HRP-C4 is in the kit: 323120**

1. Place slides back to moist chamber, check circles!
2. Add 1-5 droplets of **HRP-C4**/sample
3. **15 min** at 40C
4. Meanwhile, dilute fluorophores 1:750-1:3000 in TSA buffer, vortex and keep them at RT in dark
5. 2 min: 1x Wash at RT
6. 2 min: 1x Wash at RT
7. Place slides back to moist chamber, check circles!
8. Add 50-300 ul of vortexed RT-**TSA Plus A594**/sample
9. **30 min** at 40C
10. 2 min: 1x Wash at RT
11. 2 min: 1x Wash at RT
12. Place slides back to moist chamber, check circles!
13. Add 1-5 droplets of RT-**HRP blocker**/sample
14. **15 min** at 40C
15. 2 min: 1x Wash at RT
16. 2 min: 1x Wash at RT

## X. MOUNTING AND STORAGE

**10.** 1-3 drops **DAPI for 30 sec**
11. 1x PBS rinse
12. Mount as soon as possible after DAPI staining
13. Remove big drops of 1xPBS but do not air-dry slides
14. Add 1 small droplet of Prolong Glass antifade mountant onto each section
15. Dry slides horizontally in dry black moist chamber at 4C
16. Image preferable in 2-3 weeks, then store at -20C

## Part 3: RNAscope on vibratome slices

**Important, general comments**

- Follow promptly the protocol in the indicated order
- Never let samples dry out
- Use fresh MQ water, do not need to autoclave water
- 1 or 2 day procedures are possible
- Assay according to RNAscope® Multiplex Fluorescent Reagent Kit v2 User Manual

### XI. SAMPLE PREPARATION

- Whole animal perfusion-postfixation
  7. Work under chemical hood
  8. 1x PBS: must be cooled down, Filtered 4% PFA (or 10% NBF),
  9. For brain dissection: do not tape animals!
  10. Postfixation: ideally for 3 days at 4C
  11. Wash organs in 1x PBS
  12. Store at 4C for few days in 1x PBS or longer in 1x PBS-0.01% Na-azide

- Preparations for RNase-free sectioning:
  1. Autoclave 1 L 1x PBS
  2. Bake aluminum foil for 3 hr at 180 C
  3. Prepare fresh agarose

- Sectioning using RNase-free vibratome

  8. Fill buffer tray with 70% EtOH, rinse blades, brush, screw
  9. Use fresh 4% agarose
  10. Rinse tray with 1x PBS
  11. Trim blocks into 30 um sections
  12. Collect section in RNase-free (falcon) tubes in sterile PBS
  13. Store sections at 4 C in 1x PBS-0.01% Na-azide or in RNase-free antifreeze at -20 C

### XII. SAMPLE PRETREATMENT (DAY 1)

- $H_2O_2$-treatment (free-floating in RNase-free tubes)
  4. 1x RNase-free PBS wash for 2x15 min

5. Prepare 1% H2O2 in 1x RNase-free PBS
6. Incubate for 30 min at RT
7. 1x RNase-free PBS wash for 2x15 min
14. Place sections onto Superfrost Ultra Plus slides
8. Air dry slides at RT for 1-2 hr
9. Store at 4C

- Baking slides **(DAY 2)**
  3. Bake slide for 1 hr at 60C
  4. Leave sections at RT (optional stopping point, can be done on the previous afternoon)

- Preparations:
  3. Set oven/HybEZ oven to 40 C and 60 C
  4. Prepare Prot K buffer (0.1 M Tris-HCl, 0.05 M EDTA) and warm to 37 C
     *For 200 ml: 20 ml of 1 M Tris/HCl (pH=8), 20 ml of 0.5 M EDTA (pH=8) and 160 ml sterile MQ*
  5. Prepare 200 ml 10% NBF in blue holder – with closed lid - cool to 4 C

## XIII. SAMPLE PRETREATMENT

1. MQ wash for 2x10 min
2. Postfixation-1: 2 min at RT with pre-chilled 10% NBF (place it back to the fridge and re-use for the Postfixation-2) or with 4% PFA
3. MQ wash for 3x10 min
4. Add 100 ul Prot K (of 20 mg/ml proteinase K (EO0491, Thermo Fisher Scientific) to prewarmed 200 ml buffer, mix well
5. Prot K digestion: 20 min at 37C
6. MQ wash for 1 min
7. Postfixation-2: 2 min at RT with pre-chilled 10% NBF (collect NBF in liquid waste container) or with 4% PFA
8. MQ wash for 3x10 min
9. Make sure that Immedge PAP-PEN do not leak! Try on the glass bench
10. Draw circle on dry slides but do not let sample to dry out!
11. Air-dry for 1 min (Optional stopping point! Slides can stay at RT)
12. Keep slide in MQ until probe hybridization
13. Moist paper of HybEZ tray/black moist chambers
14. Meanwhile, prepare probe dilutions

- Probe dilutions:
  - Warm up probe stocks to 40C (10-20 min), vortex-spin
  - Dilute probes:
    - C1-probes: ready to use
    - C2, C3 probes: dilute to 1:50 in Probe diluent (PD) or in C1-probe

- Diluted can be prepared on the previous (can be store at 4 C up to 6 months)
- Right before usage, always vortex-spin/resuspend diluted probes

- Necessary ctrls:
  - Human, mouse, rat 3-plex pos ctrl
  - 3-plex neg. ctrl
  - singleplex ctrl
  - Pos. tissue ctrl
- Volumes: 50-250 ul depending on the sample size (from mouse coronal tissue to e.g. bigger human sample)

## XIV. PROBE HYBRIDIZATION

9. **Probe hybridization: 2,5 hr at 40 C in HybEZ oven**
10. Warm up 50x Wash buffer to 40 C (10 min), prepare 1X Wash in fresh MQ
    1x Wash can be stored at RT up to 1 month
    *For 2 L 1x Wash buffer: 40 ml prewarmed-50x Wash + MQ up to 2 L*
11. 2x 2 min 1x Wash at RT
12. Optinal stopping point: leave slides in 5x SSC-ben at RT in HyBEZ tray or in blue slide holder
    *For 300 ml 5x SSC: 75 ml 20x SSC + 225 ml MQ*

**20x SSC pH 7.4**

- 175 g NaCl
- 88.2 g Na-citrate
- 1L ddH2O
- adjust pH, autoclave, store at RT

## XV. SIGNAL AMPLIFICATION, CHANNEL DEVELOPMENT (DAY 2)

**Important:**

- **Never let samples dry out!**
- **Make sure that moist chamber in always wet**
- **Remove bubbles**
- **During wash steps: rock slides gently with lid on top**

- **Place AMP1, 2, 3 and HRPC1, 2, 3, HRP-blocker at RT (if slides are incubated in the HyBEZ oven. If not: prewarm the upcoming reagent to 40C)**
- **2. wash buffer can be reused as the following 1. wash buffer**
- **Make sure that the hydrophobe circle is visible before each dropping**

- **The protocol is optimized for:**
  **Channel 1: fluorescein**
  **Channel 2: Cy3**
  **Channel 3: Cy5**
  **but channels and fluorophores can be mixed.**

- Signal amplification

34. Warm up AMP1 at 40C and place AMP2, 3, HRPC1, 2, 3, HRP blocker at RT
35. 1x rinse in 1x Wash
36. Remove slides from tray/place slide back into black moist chamber
37. Add 1-4 drops of **AMP1**/sample
38. **40 min** at 40C
39. 2 min: 1x Wash at RT
40. 2 min: 1x Wash at RT

41. Remove slides from tray/place slide into black moist chamber
42. Add 1-4 drops of **AMP2**/sample
43. **40 min** at 40C
44. 2 min: 1x Wash at RT
45. 2 min: 1x Wash at RT

46. Remove slides from tray/place slide into black moist chamber
47. Add 1-4 drops of **AMP3**/sample
48. **20 min** at 40C
49. 2 min: 1x Wash at RT
50. 2 min: 1x Wash at RT

- Channel 1 development
  **Cover slides during washing steps, fluorophores are light-sensitive!**

33. Remove slides from tray/place slide into black moist chamber
34. Add 1-4 drops of **HRP-C1**/sample
35. **20 min** at 40C
36. Meanwhile, dilute fluorophores 1:750-1:3000 in TSA buffer, vortex and keep them at RT in dark
37. 2 min: 1x Wash at RT
38. 2 min: 1x Wash at RT
39. Remove slides from tray/place slide into black moist chamber
40. Add 50-250 ul of vortexed RT-**TSA Plus Fluorescein**/sample
41. **40 min** at 40C

42. 2 min: 1x Wash at RT
43. 2 min: 1x Wash at RT
44. Remove slides from tray/place slide into black moist chamber
45. Add 1-4 drops of RT-**HRP blocker**/sample
46. **20 min** at 40C
47. 2 min: 1x Wash at RT
48. 2 min: 1x Wash at RT

- Channel 2 development

16. Remove slides from tray/place slide into black moist chamber
17. Add 1-4 drops of **HRP-C2**/sample
18. **20 min** at 40C
19. 2 min: 1x Wash at RT
20. 2 min: 1x Wash at RT
21. Remove slides from tray/place slide into black moist chamber
22. Add 50-250 ul of vortexed RT-**TSA Plus Cy3**/sample
23. **40 min** at 40C
24. 2 min: 1x Wash at RT
25. 2 min: 1x Wash at RT
26. Remove slides from tray/place slide into black moist chamber
27. Add 1-3 drops of RT-**HRP blocker**/sample
28. **20 min** at 40C
29. 2 min: 1x Wash at RT
30. 2 min: 1x Wash at RT

- Channel 3 development

16. Remove slides from tray/place slide into black moist chamber
17. Add 1-3 drops of **HRP-C3**/sample
18. **20 min** at 40C
19. 2 min: 1x Wash at RT
20. 2 min: 1x Wash at RT
21. Remove slides from tray/place slide into black moist chamber
22. Add 50-250 ul of vortexed RT-**TSA Plus Cy5**/sample
23. **40 min** at 40C
24. 2 min: 1x Wash at RT
25. 2 min: 1x Wash at RT

26. Remove slides from tray/place slide into black moist chamber
27. Add 1-3 drops of RT-**HRP blocker**/sample
28. **20 min** at 40C
29. 2 min: 1x Wash at RT
30. 2 min: 1x Wash at RT

## XVI. IHC (primary antibody)

1. 15 min wash in PBS
2. Add primary antibody
3. Close the box (wet humidity chamber) to prevent the sections from drying out, store it overnight at RT (if the primary antibody should be on for two days, place it on the second day at 4 degrees).

## XVII. IHC (secondary antibody)

1. 2x15 min wash in PBS
2. Add secodary antibody
3. Close the box (wet humidity chamber) to prevent the sections from drying out, store it 3 hours at RT
4. 2x15 min wash in PBS

## XVIII. MOUNTING AND STORAGE

17. 1-3 drops DAPI for 3 min
18. 1x PBS rinse
19. Mount as soon as possible after DAPI staining
20. Remove big drops of 1xPBS but do not air-dry slides
21. Add 1 drop Prolong Glass antifade mountant onto sample
22. Dry slides horizontally in dry black moist chamber at 4C
23. Image preferable in 2-3 weeks
24. Store at -20C