

## ***In situ* Hybridization of miRNAs in Human Embryonic Kidney and Human Pluripotent Stem Cell-derived Kidney Organoids**

Filipa M. Lopes, Susan J. Kimber and Ioannis Bantounas\*

Division of Cell Matrix Biology and Regenerative Medicine, Faculty of Biology, Medicine and Health, University of Manchester, and the Manchester Academic Health Science Centre, Manchester, UK

\*For correspondence: [ioannis.bantounas@manchester.ac.uk](mailto:ioannis.bantounas@manchester.ac.uk)

**[Abstract]** MicroRNAs are small RNAs that negatively regulate gene expression and play an important role in fine-tuning molecular pathways during development. There is increasing interest in studying their function in the kidney, but the majority of studies to date use kidney cell lines and assess the total amounts of miRNAs of interest either by qPCR or by high-throughput methods such as next generation sequencing. However, this provides little information as to the distribution of the miRNAs in the developing kidney, which is crucial in deciphering their role, especially as there are multiple kidney cell types, each with its own specific transcriptome. Thus, we present a protocol for obtaining spatial information for miRNA expression during kidney development by *in situ* hybridization (ISH) of anti-miRNA, digoxigenin-labelled (DIG), Locked Nucleic Acid (LNA<sup>®</sup>) probes on (i) native human embryonic tissue and (ii) human pluripotent stem cell (hPSC)-derived 3D kidney organoids that model kidney development. We found that the method reveals the precise localization of miRNA in specific anatomical structures and/or cell types and confirms their absence from others, thus informing as to their specific role during development.

**Keywords:** Kidney development, Organoids, hESC, hPSC, MicroRNA, *In situ* hybridization, LNA probe

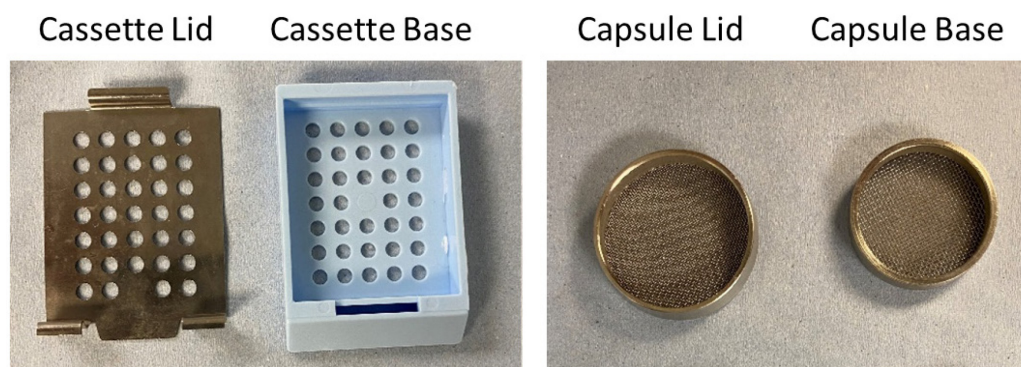
**[Background]** MicroRNAs (miRNAs) are small (20-25 nucleotides) RNAs that regulate gene expression by binding predominantly to the 3' UTRs of their target genes' mRNAs and inhibiting their translation and/or causing their degradation (Bartel, 2018). An increasing number of studies now show that miRNAs play a crucial role in fine-tuning molecular pathways in the kidney, both in normal development and in disease (Jones *et al.*, 2018; Trionfini *et al.*, 2015; Zhao *et al.*, 2019). The majority of these studies have been conducted on animal models, which do not always faithfully recapitulate the developmental events or disease phenotypes in humans. To overcome these limitations, we recently reported using human pluripotent stem cell (hPSC)-derived kidney organoids (Bantounas *et al.*, 2018; Takasato *et al.*, 2015) as a model to study the role of the *miR-199a/214* cluster during human kidney development (Bantounas *et al.*, 2021). In that study, we used *in situ* hybridization of miRNAs with digoxigenin-labelled locked-nucleic acid (LNA<sup>®</sup>) probes to detect the precise localization of the miRNAs of this cluster in paraffin sections of both native kidney and organoids. LNA<sup>®</sup> probes have a chemically modified backbone to afford a higher melting temperature ( $T_m$ ) per nucleotide than conventional DNA or RNA probes (Singh *et al.*, 1998). They are, thus, ideally suited to the detection of smaller RNAs for which conventional probes would have too low a  $T_m$  to bind efficiently. After binding to the tissue, the probes are detected

by application of an alkaline phosphatase (AP)-linked, anti-digoxigenin antibody, followed by the addition of an AP substrate, which is converted to a coloured product, visible under a light microscope. Using this method, we discovered that *miR-199a-3p* and *miR-214-3p* were both present in the kidney stroma and in developing glomeruli but were largely absent from mature glomeruli. One of the miRNAs, *miR-214-3p*, also exhibited strong tubular expression. In addition, we observed differences in the extent and distribution of expression between embryonic/fetal kidney and organoids (particularly in the case of *miR-199a-3p*), possibly indicating that the two represented different developmental stages (Bantounas *et al.*, 2021). This example demonstrates the main advantage of this method, which is the acquisition of spatial information regarding the expression of the miRNAs of interest within a given tissue. This information would be lost if one were to rely exclusively on transcriptional detection methods [e.g., qPCR or Next Generation miRNA Sequencing (miRseq)]. The hybridization steps of the protocol presented here (see below) were adapted from Jørgensen *et al.* (2010) and were optimized for kidney/kidney organoids and the particular miRNAs we studied (Bantounas *et al.*, 2021). However, the method can in principle be used with formalin-fixed, paraffin-embedded (FFPE) sections of any tissue and for any miRNA or other small RNA of similar size.

## Materials and Reagents

1. 1.5 ml Eppendorf tubes (Starlab, catalog number: S1615-5550)
2. Plastic pipette tips (preferably filtered) (Starlab, catalog numbers: S1122-1830 [1,000 µl]; S1120-8810 [200 µl]; S1123-1810 [20 µl]; S1121-3810 [10 µl]; or equivalent)
3. Vacuum filter units, pore size 0.2 µm (Thermo, catalog number: 568-0020)
4. 6-well plates (Costar, catalog number: 3516, or equivalent)
5. 3 ml plastic Pasteur pipettes (Starlab, catalog number: E1414-0311, or equivalent)
6. Tissue processing/embedding cassettes (Simport, catalog number: M490-4) (Figure 1, Left)
7. Stainless steel or plastic tissue processing capsules (Figure 1, Right)

*Note: The capsules depicted in Figure 1 were the ones used by the authors but have now been discontinued. Therefore, alternative options are proposed here: Simport, catalog number: M470; or Fisher, catalog number: 15-182-219; or equivalent.*



**Figure 1. Tissue processing cassette and capsule**

8. Metal molds for paraffin embedding of tissue (Leica, catalog number: 3803081E)
9. RNaseZap® wipes for disinfection of working surfaces (Invitrogen/Ambion, catalog number: AM9788)
10. Superfrost® Plus slides (ThermoFisher Scientific, catalog number: J1800AMNZ)
11. Human embryonic kidney tissue was provided by the MRC and Wellcome Trust Human Developmental Biology Resource (<http://www.hdbr.org/>)
12. hPSC-derived kidney organoids can be produced according to published protocols (Takasato *et al.*, 2015; Bantounas *et al.*, 2018 and 2021). In our study (Bantounas *et al.*, 2021), we differentiated the MAN13 human embryonic stem cell (hESC) line (Ye *et al.*, 2017), but any hESC or induced pluripotent stem cell (iPSC) line can be used in principle
13. Paraffin wax for embedding tissue, melting point 61°C (Pfm Medical, catalog number: 9000)
14. Nuclease-free water (not DEPC-treated) (ThermoFisher Scientific, catalog number: AM9930)
15. Phosphate buffered saline (PBS), without Ca<sup>2+</sup> and Mg<sup>2+</sup> (Sigma, catalog number: D8537-500 ml)
16. 5'-Digoxigenin-labelled miRCURY® LNA® miRNA detection probes (1 nmol) (see Note 1) (QIAGEN, catalog number: 339111; see below for individual probe codes). In this example, we used probes targeting:
  - a. *miR-199a-3p* (QIAGEN, catalog number: YD00615410)
  - b. *miR-214-3p* (QIAGEN, catalog number: YD00611471)
  - c. scrambled probe (negative control; QIAGEN, catalog number: YD00699004)
  - d. U6 snRNA (positive/optimization control; QIAGEN, catalog number: YD00699002)
17. miRCURY® LNA® miRNA ISH Buffer Set (FFPE) (QIAGEN, catalog number: 339450), which includes:
  - a. 2× Formamide-free miRNA ISH buffer
  - b. Proteinase K solution
18. UltraPure™ 20× SSC Buffer (Invitrogen, catalog number: 15557-044)
19. Tween-20 (Sigma, catalog number: P1379-250ML)
20. Sheep anti-DIG-AP (alkaline phosphatase linked) antibody (Roche/Sigma-Aldrich, catalog number: 11093274910)
21. Sheep serum (Sigma-Aldrich, catalog number: S3772-10ML)
22. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A1470-100G)
23. Nitro blue tetrazolium/5-bromo-4-chloro-indolyl-phosphate (NBT/BCIP) ready-to-use tablets (Roche, catalog number: 1 1 697 471 001)
24. Levamisole (Fluka, catalog number: 31742), diluted in water to make a 100 mM stock solution
25. Nuclear Fast Red solution (Merck, catalog number: N3020)
26. Industrial methylated spirit (IMS) (various suppliers)
27. Xylene (various suppliers)
28. 100% Ethanol (various suppliers)

29. KCl (various suppliers)
30. KP Marker Plus (Histolab, catalog number: 98307-R) or similar hydrophobic marking pen (various suppliers)
31. 4% Paraformaldehyde (PFA) Solution (see Recipes)
32. 1 M Tris Buffer (see Recipes)
33. 0.5 M EDTA Solution (see Recipes)
34. 5 M NaCl Solution (see Recipes)
35. 1× Proteinase K Buffer (see Recipes)
36. SSC Buffer dilutions (see Recipes)
37. PBS-T 0.1% (see Recipes)
38. Blocking solution (see Recipes)
39. Antibody dilution buffer (see Recipes)
40. KTB buffer (see Recipes)

## **Equipment**

1. Tissue Processor (Leica, model: ASP300S; or equivalent)
2. Heated Paraffin Embedding Station (Leica HistoCore Arcadia H or equivalent)
3. Cold Plate (Leica, HistoCore Arcadia C or equivalent)
4. Microtome (Leica, model: RM225; or equivalent)
5. Hybridization oven or other variable temperature incubator (e.g., Hybaid, Midi Dual 14 or equivalent)
6. Microscope slide rack (Simport, catalog number: M905-12DGY)
7. Jars for immersing the slide rack into solvents/buffers (Simport, catalog number: M900-12G)
8. Heating block (up to 90°C) (Eppendorf ThermoMixer F1.5 or equivalent)
9. Microcentrifuge (Labnet, Prism or equivalent)
10. Gilson® or equivalent pipettes (various suppliers)
11. Staining tray for holding the slides during hybridization and staining (see Figure 2).  
*Note: The tray depicted in Figure 2 was the one used by the authors but has now been discontinued. An alternative is proposed here: Fisher, catalog number: 22-045-035.*
12. Waterbath (Grant SUB6, catalog number: P266; or equivalent)
13. Autoclave (various suppliers)
14. Optionally, for imaging: 3D-Histech Panoramic-250 microscope slide-scanner, with a 40×/0.95 Plan Apochromat objective (Zeiss)

## **Software**

1. Optional: CaseViewer (3DHISTECH Ltd.; [www.3dhitech.com](http://www.3dhitech.com)), to capture images following scanning with a 3D-Histech slide-scanner (see point 14, in Equipment)

2. Fiji/ImageJ (<http://imagej.net/Fiji/Downloads>) (for image processing following capture)

## **Procedure**

### **A. Tissue Fixation**

The procedure described in this section is for preparing organoid tissue and is an adaptation of the one described in Lopes *et al.* (2019).

**If beginning with samples already embedded in paraffin: Skip directly to section B, step 10.**

The procedure below assumes organoids cultured in transwell inserts in 6-well plates (Bantounas *et al.*, 2018). If a different size well and/or insert is used, change volumes proportionally in Steps A1-A7.

1. Prepare “wash” and “fixation” 6-well plates, allowing one well per transwell insert of organoids:
  - a. Wash plate(s): Add 1.2 ml PBS into each well.
  - b. Fixation plate(s): Add 1.2 ml of 4% PFA into each well.
2. Transfer the transwell inserts from the culture plate to the wash plate prepared above.
3. Carefully add 1 ml of PBS into each transwell and swirl the plate gently to wash the organoids.
4. Using a Gilson or a 3 ml Pasteur pipette, remove the PBS from inside the transwells.
5. Transfer the transwells from the “wash” to the “fixation” plate (*i.e.*, on top of the PFA that is already in each of the wells).
6. Add 1 ml 4% PFA into each transwell making sure that the organoids are fully covered.  
*Note: You may add more PFA, if necessary, to completely cover the organoids.*
7. Incubate at room temperature for 20 min.
8. Remove the PFA from the transwells and transfer them to a new wash plate, prepared as in Step A1a, above.
9. Wash the organoids twice with PBS by repeating Steps A2-A4 as above.
10. Using a 3 ml plastic Pasteur pipette, take some of the PBS of the last wash in the transwell and expel it onto each organoid, with just enough force so as to dislodge it (but take care not to be too forceful, which can break up the organoid).
11. Using the same 3 ml Pasteur, transfer each organoid into a separate fresh 1.5 ml Eppendorf tube containing 400  $\mu$ l PBS. The fixed organoids can be stored at 4°C in PBS for up to one week before embedding.

### **B. Embedding fixed tissue in paraffin and sectioning**

A visual guide to the embedding and sectioning of tissues, which is also applicable to our organoids in this section, can be found at this address: <https://www.youtube.com/watch?v=7-LIbAWPc-g> (accessed in 2021).

1. Label a number of plastic tissue embedding cassettes (point 8, in Materials and Reagents; Figure 1, Left) equal to the number of organoids with a pencil or permanent marker pen to distinguish between samples.

2. Place a tissue processing capsule (point 9, in Materials and Reagents; Figure 1, Right) into each plastic tissue cassette, both with lids off.
3. Using a plastic Pasteur pipette, carefully transfer one organoid into the capsule base.
4. Replace the lid on the tissue processing capsule, followed by the lid on the tissue cassette.
5. Immerse the cassette into 70% ethanol.
6. Repeat Steps B3-B5 for each organoid.
7. Transfer the cassettes to the tissue processor (Leica ASP300S or equivalent) and run a program following the steps as listed in Table 1.

**Table 1. Tissue processing program (R.T.: room temperature)**

Step/Solvent	Time	Temperature
70% IMS	20 min	R.T.
70% IMS	30 min	R.T.
90% IMS	45 min	R.T.
90%IMS	60 min	R.T.
100% Absolute Alcohol	30 min	R.T.
100% Absolute Alcohol	45 min	R.T.
100% Absolute Alcohol	60 min	R.T.
Xylene	20 min	40°C
Xylene	30 min	40°C
Xylene	40 min	40°C
Wax	70 min	61°C
Wax	70 min	61°C
Wax	70 min	61°C

8. The next day, take the cassettes out of the tissue processor and transfer them into paraffin wax, using a Heated Paraffin Embedding Station, as follows:
  - a. Open the plastic cassette followed by the tissue processing capsule.
  - b. With the aid of liquid wax from the embedding station, carefully push the organoid into a metal mold.
  - c. Carefully position the organoid in the middle of the mold.
  - d. Allow to set for a few seconds on a Cold Plate (see Equipment). Top up the mold with liquid wax, place the plastic cassette, without lid, on the top of the mold and leave it to solidify on the cold plate for about 2 h.
9. Repeat Steps B8a-B8d for each organoid.
10. Place the blocks in a container of melting ice/icy water for at least 15 min before sectioning and using a microtome, produce serial 5 µm sections of the paraffin block containing the organoid.
11. Allow the tissue sections to extend (so that any creases are straightened out) on the surface of clean warm tap water and then transfer them to labelled microscope slides. Allow to dry out on a warm plate and transfer to a 37°C oven overnight for further drying.



### C. Deparaffinization of tissue sections

*Caution: All steps in this section should be performed in a chemical hood.*

Prior to starting the procedure, prepare a series of eleven jars containing the solvent solutions mentioned in each of the steps below.

1. Place the slides in a slide rack and immerse into a jar of xylene for 5 min.
2. Immerse in a 2<sup>nd</sup> jar of xylene for 5 min.
3. Immerse in a 3<sup>rd</sup> jar of xylene for 5 min.
4. Immerse 10 times (2-3 s each time) in a jar of 99.9% ethanol.
5. Immerse 10 times (2-3 s each time) in a 2<sup>nd</sup> jar of 99.9% ethanol.
6. Immerse in a 3<sup>rd</sup> jar of 99.9% ethanol for 5 min.
7. Immerse 10 times (2-3 s each time) in a jar of 96% ethanol.
8. Immerse in a 2<sup>nd</sup> jar of 96% ethanol for 5 min.
9. Immerse 10 times (2-3 s each time) in a jar of 70% ethanol.
10. Immerse in a 2<sup>nd</sup> jar of 70% ethanol for 5 min.
11. Immerse in a jar of PBS for 5 min.

### D. Proteinase K treatment

The Proteinase K concentration and length of treatment presented below were optimized for PFA-fixed, paraffin-embedded human embryonic kidney tissue and kidney organoids. For different fixation methods and/or tissue types, these parameters need to be re-optimized (see Note 2).

1. Add 0.75 µl Proteinase K stock solution per ml of Proteinase K buffer (see Recipe 5) to obtain 1× Proteinase K reagent. For our fixation/tissue combination (Bantounas *et al.*, 2021), we determined the optimum concentration to be 0.33×; therefore, further dilute the 1× Proteinase reagent 1 in 3, with Proteinase K buffer (see Note 2). Make enough working solution for approximately 300 µl of final reagent per section to be stained (although less may be enough in the case of the organoids).
2. With the slides on a flat surface, apply 300 µl (or enough to cover the section) of Proteinase K reagent directly onto each section.
3. Transfer the slides to a pre-heated hybridization oven and incubate at 37°C for 10 min (see Note 2).
4. Place the slides onto a slide rack and wash twice by immersing into a jar of PBS.

### E. Probe hybridization

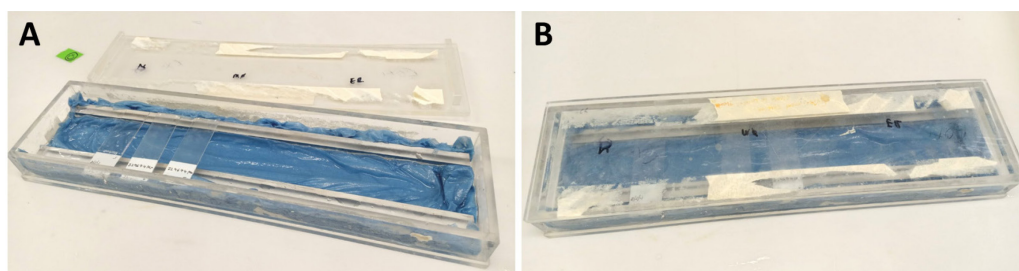
The final probe concentrations and hybridizing temperature given below are specific for the miRNAs we studied in Bantounas *et al.* (2021) and are given as examples. These parameters should be optimized separately for each individual miRNA (see Note 3). We recommend that alongside the miRNA of interest, a negative control (scrambled) probe, as well as a positive (U6 snRNA) probe is used (see also Table 2).

1. Dilute the 2× Formamide-free miRNA ISH buffer with an equal volume of RNase-free water to obtain 1× miRNA ISH buffer. Prepare enough for all probe/section combinations (see Step E2).
2. In an RNase-free Eppendorf tube, dilute the probe stock in 1× miRNA ISH buffer appropriately to obtain the desired working/final concentration LNA<sup>®</sup> probe mix (see Note 3). Table 2 shows examples of probes optimized at different final concentrations as used in Bantounas *et al.* (2021). The final volume should be enough to add 50 µl per section (although you can scale up or down depending on the size of your section).

**Table 2. Examples of stock and final concentrations of LNA<sup>®</sup> probes used on kidney tissue/organoids**

Probe	Stock concentration	Final Concentration	Dilution Factor
U6 snRNA (+ve control)	0.5 µM	0.1 nM	1: 5,000
miR-199a-3p	25 µM	40 nM	1: 625
miR-214-3p	25 µM	1 nM	1: 25,000
Scrambled (-ve control)	25 µM	40 nM	1: 625

3. Place the tubes in a heating block, at 90°C for 4 min, to denature the probes. Then, centrifuge briefly to collect all the liquid at the bottom of the tube.
4. Prepare a slide holding staining tray by placing wet tissue at the bottom and place the slides on top, as shown in Figure 2A.
5. Apply 50 µl of LNA<sup>®</sup> probe mix onto each section and replace the lid on the staining tray (Figure 2B).



**Figure 2. Set up of slides on staining tray, with wet tissue underneath to prevent evaporation of the applied solution during hybridization**

6. Place the slide box in the oven at 55°C (see Note 3) for 1 h.
7. Remove the slide box from the oven, place the slides in a slide rack and immerse in a jar containing 5× SSC buffer (Recipe 6).
8. Prepare a series of six jars containing different concentrations of SSC buffer (see Recipe 6), as detailed below in Table 3, and wash the slides by serially immersing in each of them for 5 min. Keep the jars in a waterbath set at the hybridization temperature, except the last one (#6), which should be at room temperature.



**Table 3. Stringent washes following probe hybridization**

Wash #	Buffer
1	5× SSC
2	1× SSC
3	1× SSC
4	0.2× SSC
5	0.2× SSC
6	0.2× SSC

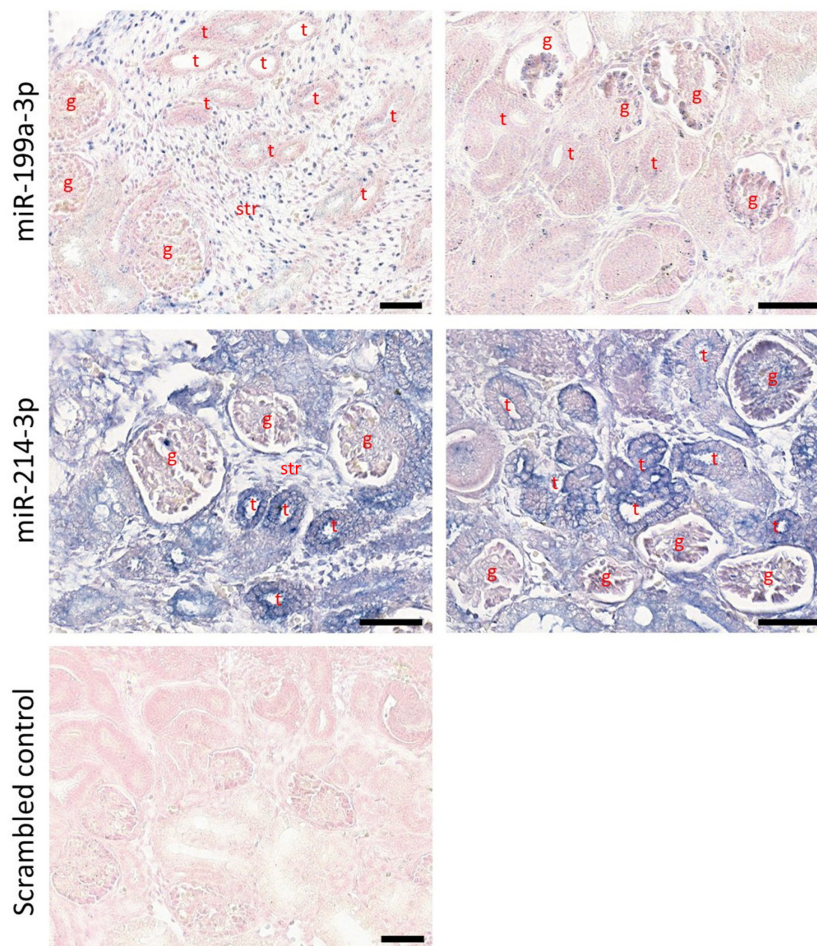
- Transfer the slides into a jar containing PBS.

**F. Antibody-detection and visualization of bound probe**

*Caution: In the blocking and immunostaining steps below, do not let the tissue sections dry out, as this can lead to a very high background signal and also risks compromising the integrity of the tissue.*

- Using the KP marker (see Materials), apply a hydrophobic barrier around each section.
- Add 50 µl (or enough to cover the section) blocking solution (Recipe 8) per section and incubate at room temperature for 15 min.
- During blocking, dilute the anti-digoxigenin (anti-DIG-AP) antibody 1:400 (see Note 4) in antibody dilution buffer (Recipe 9), allowing 50 µl (or enough to cover the section) per section.
- Remove the blocking solution by tipping the slides, then apply the antibody to the sections and incubate with the antibody at room temperature for 1 h.
- Wash the slides three times by placing them in a slide rack and successively immersing in three jars containing fresh PBS-T 0.1% (Recipe 7) for 3 min each.
- To make the AP reaction substrate, dissolve an NBT-BCIP tablet in 10 ml distilled water. Then, add 20 µl Levamisole stock solution (see Recipes) (2 µl per ml of NBT-BCIP solution).  
*Caution: The substrate solution must be used immediately and be protected from light.*
- Apply 50 µl (or enough to cover the section) substrate per section and incubate in the dark for 2 h in a 30°C oven (see Note 5).
- To stop the reaction, place the slides in a slide rack and immerse in a jar containing KTBT buffer (Recipe 10) for 5 min.
- Repeat the above wash a second time.
- Wash the slides with distilled water twice, for 1 min each.
- Apply 50 µl (or enough to cover the section) Nuclear Fast Red counterstain for 1 min.
- Place the slides in a slide rack and into a jar containing tap water. Rinse under running tap water for 10 min.
- The slides can be examined and imaged using a brightfield microscope. MiRNA-positive cells will be stained blue. Optionally, to capture the entire section in detail, use a 3D-Histech Panoramic-250 microscope slide-scanner. Subsequently, images can be analysed and

snapshots captured with the CaseViewer software and further processed using ImageJ. Representative results can be seen in Figure 3.



**Figure 3. Detection of miRNAs in human fetal kidney sections (week 12 of gestation) by *in situ* hybridization with digoxigenin-labelled LNA<sup>®</sup> probes.** *Mir-199a-3p* was present in the stroma and immature glomeruli, while *miR-214-3p* was present in the stroma, immature glomeruli and tubules (g: glomerulus; t: tubule; str: stroma). Scale bars are 50  $\mu$ m.

## Notes

1. QIAGEN recommends the use of double-labelled (both 5' and 3') digoxigenin probes for miRNA detection. However, we found that in our tissue/miRNA combination, single-labelled (5') probes gave good results at a lower cost. It is possible that for other tissue/miRNA combinations, a double-labelled probe is indeed necessary (e.g., for less abundant miRNAs).
2. This protocol assumes PFA fixation and paraffin embedding of tissue sections. If either of these conditions changes, Proteinase K concentration and duration of treatment may need to be re-optimized, even for the same tissue type. In such cases, a pilot experiment must be carried out using the positive control (U6) LNA<sup>®</sup> probe, varying the range of Proteinase K reagent between

0.03× and 2× and incubating for 10 min. Then, use the concentration that gave the best results and perform a second pilot experiment, varying the duration of treatment between 5 min and 30 min.

3. The optimal LNA® probe concentration is miRNA-specific, and the optimal hybridization temperature is usually around 30°C below its  $T_m$  (as given by the manufacturer). Pilot experiments can be performed for each probe, varying the concentration between 5 nM and 80 nM (although, occasionally, the optimum could be even lower than this; *e.g.*, the *miR-214-3p* probe in our study was used at 1 nM; Bantounas *et al.*, 2021). The 55°C, mentioned in our protocol, is usually a good starting point as a hybridization temperature, but the optimum can be determined in subsequent pilot experiments, and it usually falls into the 50°C to 60°C range.  
*Note: It is possible that no positive results are initially obtained in the probe concentration optimization step, in which case, you should optimize the temperature first using a mid-range probe concentration (e.g., 10-20 nM), following which you should refine the concentration further.*
4. If desired, the antibody dilution can be optimized by using dilutions in the range of 1:200-1:2,000.
5. If a 2 h development time results in too strong or too weak a signal, you could vary the incubation time of the AP substrate. With some tissue/miRNA combinations, you may be able to observe the colour as it develops under a light microscope and stop or prolong the incubation time accordingly.

## **Recipes**

1. 4% Paraformaldehyde (PFA) Solution
  - a. To a glass beaker, add 800 ml of 1× PBS and heat to 60°C, with stirring on a heated stir plate, in a chemical hood.
  - b. Then add 40 g PFA. Raise the pH by adding 10 M NaOH dropwise until the PFA goes into solution (solution becomes clear).
  - c. Allow the solution to cool to room temperature and filter (*e.g.*, using a vacuum filter unit).
  - d. Adjust the volume to 1,000 ml with 1× PBS.
  - e. Check the pH again and adjust with small amounts of 1 M HCl to 6.9.
  - f. Store in 10 ml aliquots at -20°C. Defrosted aliquots can be stored at 4°C for up to one month.
2. 1 M Tris Buffer
  - a. In 800 ml distilled water, dissolve 121.1 g Tris Base.
  - b. Adjust pH to 7.4 by adding concentrated (37% w/v) HCl (approximately 70 ml).
  - c. Make sure the solution is at room temperature before making final adjustments to pH, then adjust the total volume to 1,000 ml before autoclaving. Store indefinitely at room temperature.
3. 0.5 M EDTA Solution
  - a. To 800 ml of distilled water, add 186.1 g disodium-EDTA·2H<sub>2</sub>O.
  - b. While stirring using a magnetic stirrer, adjust the pH to 8, using NaOH. Bring volume to

- 1,000 ml with water.
- c. Autoclave and store at room temperature. Discard if EDTA precipitates out of solution.
4. 5 M NaCl Solution
    - a. Dissolve 292.2 g of NaCl in 800 ml distilled water.
    - b. Bring volume to 1,000 ml with water.
    - c. Autoclave and store indefinitely at room temperature.
  5. 1× Proteinase K Buffer

To 900 ml RNase free water, add:

5 ml of 1 M Tris-HCl, pH 7.4

2 ml 0.5 M EDTA

0.2 ml 5 M NaCl

Bring volume to 1,000 ml, then autoclave. Store indefinitely at room temperature.
  6. SSC Buffer dilutions

5× SSC: Add 250 ml of 20× SSC to 750 ml distilled water

1× SSC: Add 50 ml of 20× SSC to 950 ml distilled water

0.2× SSC: Add 10 ml of 20× SSC to 990 ml distilled water

Autoclave and store indefinitely at room temperature.
  7. PBS-T 0.1%

To 1 L of 1× PBS, add 1 ml Tween-20. Store at room temperature for up to one month.
  8. Blocking solution (prepare fresh each time)

Per 1 ml of PBS-T 0.1% (see Recipe 7 above), add 20 µl sheep serum (2% v/v) and 10 mg BSA (1% w/v). Scale up as necessary.
  9. Antibody dilution buffer (prepare fresh each time)
    - a. Prepare PBS-T 0.05% by mixing equal volumes of PBS-T 0.1% (see Recipe 7 above) with PBS.
    - b. Then, per 1 ml of the resultant solution, add 10 µl sheep serum (1% v/v) and 10 mg BSA (1% w/v).
  10. KTB buffer  
To 900 ml RNase free water, add:

7.9 g Tris-HCl

8.7 g NaCl

0.75 g KCl

Bring volume to 1,000 ml with water and autoclave. Store indefinitely at room temperature.

## **Acknowledgments**

The protocol is presented as used in the original research paper (Bantounas *et al.*, 2021), in which the probe-binding and visualizing part is an adaptation, with some modifications, of the protocol recommended in QIAGEN's miRCURY® LNA® miRNA Detection Probes Handbook (October 2017

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### **Competing interests**

The authors declare no competing interests.

### **Ethics**

Human tissues, collected after maternal consent and ethical approval (REC 08/H0906/21+5), were provided by the MRC and Wellcome Trust Human Developmental Biology Resource (<http://www.hdbi.org/>).

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