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AAV-Zombie on tissue sections

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We use this protocol and it's working

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Abstract

Detection of AAV genomes *in situ* can facilitate understanding of AAV transduction and processing. This protocol enables spatial detection of AAV genomes and concatemers in tissue and is based on the Zombie method published in Askary et al., *Nat Biotechnol 38*: 66. AAVs carrying barcoded genomes are delivered to cells. Following fixation, a phage RNA polymerase is used to transcribe the barcode *in situ,* yielding barcoded transcripts that can be detected with RNA FISH. This protocol is compatible with either ssAAV or scAAV genomes. In the former case, detected genomes will be primarily nuclear and detectable once second strand synthesis has occurred. In the latter case, the genome can be detected regardless of where in the transduction pathway the genome is. This protocol can be combined with RNA FISH detection of reporter transcripts or endogenous transcripts, enabling measurement of expression and cell type classification.

Materials

- Methanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**322415
- Acetic Acid 100% **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A6283
- Ethanol, anhydrous **VWR International Catalog #**89125-172
- UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #**10977023
- Paraformaldehyde 32% (methanol free) **Electron Microscopy Sciences Catalog #**15714
- PBS - Phosphate-Buffered Saline (10X) pH 7.4 **Thermo Fisher Scientific Catalog #**AM9625
- SSC (20X), RNase-free **Thermo Fisher Catalog #**AM9763
- Ethylene carbonate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**E26258
- Dextran Sulfate, 50% solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**3730-OP
- Tween 20, 10% aqueous solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**11332465001
- MEGAscript™ T7 Transcription Kit **Thermo Fisher Scientific Catalog #**AMB13345
- MEGAscript™ SP6 Transcription Kit **Thermo Fisher Scientific Catalog #**AM1330
- Hoechst 33342, Trihydrochloride, Trihydrate - 10 mg/mL Solution in Water **Thermo Fisher Scientific Catalog #**H3570
- ProLong Diamond Antifade Mountant **Thermo Fisher Scientific Catalog #**P36970
- Tissue-Plus™ O.C.T. Compound **Fisher Scientific Catalog #**23-730-571
- Phosphate buffered saline powder, pH 7.4, for preparing 1 L solutions **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P3813
- Heparin sodium salt from porcine intestinal mucosa **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**H3149

HCR FISH probes - can be obtained as ssDNA oligos, from Integrated DNA Technologies (IDT; **https://www.idtdna.com/**) and/or from Molecular Instruments (**https://www.molecularinstruments.com/**)
HCR hairpins - can be obtained from Molecular Instruments (**https://www.molecularinstruments.com/**)
Fluorophore-conjugated polyT probes can be obtained from Integrated DNA Technologies (IDT; **https://www.idtdna.com/**)

For paired IHC and AAV-Zombie, you will also need:

- BlockAid™ Blocking Solution **Thermo Fisher Scientific Catalog #**B10710
- Triton X-100, 10% solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**93443

Suitable primary and secondary antibodies (e.g.

- anti-AAV VP1/VP2/VP3 mouse monoclonal, B1, AFDye™ 488 Conjugate **PROGEN Catalog #**61058-488)

Note

We have successfully detected capsid proteins with immunofluorescence staining via a monoclonal antibody against linear capsid epitopes (clone B1). As MAA fixation is expected to denature proteins, we suggest trying primary antibodies against linear epitopes vs. those against conformational epitopes.

Safety warnings

- AAVs are biohazardous materials and must be handled according to governmental and institutional regulations. Experiments involving AAVs were performed using biosafety level 2 practices as required by the California Institute of Technology and the US Centers for Disease Control and Prevention.
- rAAVs, although replication-incompetent, are potent gene-delivery vehicles and must be handled according to governmental and institutional regulations. The safety of packaged transgenes (e.g., oncogenic genes) should be carefully considered. Perform all procedures in a certified biosafety cabinet and clean AAV-contaminated equipment, surfaces, and labware with fresh 10% (vol/vol) bleach.
- AAVs are biohazardous materials and must be handled according to governmental and institutional regulations. All experiments involving the aforementioned materials were performed in a Class II biosafety cabinet with annual certification as required by the California Institute of Technology and the US Centers for Disease Control and Prevention.

Ethics statement

Animal husbandry and all procedures involving animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee (IACUC) and by the Office of Laboratory Animal Resources at the California Institute of Technology.

Before start

This protocol enables spatial detection of AAV genomes and concatemers in tissue. For detection of AAV genomes, a Zombie barcode and phage RNA polymerase promoter are required in a single AAV genome. For detection of AAV concatemers (SpECTr), 2 AAV genomes are required: one genome carries a Zombie barcode and the other carries a phage RNA polymerase promoter. Barcode sequences can be found in Supplementary Table 1 of Askary et al., *Nat Biotechnol 38*: 66.

See https://www.biorxiv.org/content/10.1101/2023.12.23.573214v1 for more information on these methods.

Reagent set up

1 General note on reagents and consumables

> **Note**
>
> As AAV-Zombie detects AAV genomes through an RNA transcript proxy, it's key that all reagents, tools, and work surface are RNase-free. Use RNase-free consumables and reagents to prepare all buffers.

2 Prepare 3:1 methanol:acetic acid (MAA) fixation buffer by combining:

- Methanol: 180 mL
- Glacial acetic acid: 60 mL

in a 250 mL sterile bottle. Store solution at -20 °C for up to 3 months.

> **Note**
>
> Scale up or down volumes based on needs.

3 Prepare 70% ethanol solution, by combining

- 100% Ethanol: 175 mL
- UltraPure water: 75 mL

in a 250 mL sterile bottle. Store solution at -20 °C for up to 3 months.

> **Note**
>
> Scale up or down volumes based on needs.

4 Prepare 4% paraformaldehyde, 1x PBS solution (4% PFA, 1x PBS), by combining:

- 32% paraformaldehyde: 25 mL
- 10x Phosphate-buffered saline (PBS): 20 mL
- UltraPure water: 155 mL

in a 250 mL sterile bottle. Store solution at 4 °C for up to 1 month.

5 Prepare probe hybridization buffer, by combining:

- 20x saline-sodium citrate (SSC) buffer: 5 mL
- Ethylene carbonate: 5 mL
- 50% dextran sulfate: 10 mL
- UltraPure water: 30 mL

in a 50 mL conical tube. Store solution at Room temperature for up to 3 weeks, or -20 °C for up to a year.

Note

Scale up or down volumes based on needs. We make appropriately sized aliquots (e.g. 10 mL) and store at -20 °C .

Ethylene carbonate is a non-toxic substitute for formamide, and may also help to reduce background from non-specific interaction (as compared to formamide-based hybridization buffers). Substituting ethylene carbonate for formamide, at the same concentration, should yield qualitatively similar results.

Ethylene carbonate is solid at room temperature. Pre-warm to 37 °C to melt.

Probe hybridization buffer can also be obtained from Molecular Instruments (**https://www.molecularinstruments.com/**)

6 Prepare stringent wash buffer, by combining:

- 20x saline-sodium citrate (SSC) buffer: 5 mL
- Ethylene carbonate: 15 mL
- UltraPure water: 30 mL

in a 50 mL conical tube. Store solution at Room temperature for up to 3 weeks, or -20 °C for up to a year.

Note

Scale up or down volumes based on needs. We make appropriately sized aliquots (e.g. 10 mL) and store at -20 °C .

Ethylene carbonate is a non-toxic substitute for formamide, and may also help to reduce background from non-specific interaction (as compared to formamide-based wash buffers). Substituting ethylene carbonate for formamide, at the same concentration, should yield qualitatively similar results.

Stringent wash buffer can also be obtained from Molecular Instruments (as "Probe wash buffer"; **https://www.molecularinstruments.com/**)

7 Prepare wash buffer (5x SSC, with 0.1% Tween-20), by combining:

- 20x saline-sodium citrate (SSC) buffer: 100 mL
- 10% Tween-20: 5 mL
- UltraPure water: 395 mL

Store solution at Room temperature for up to a year.

8 Prepare 5x SSC, by combining:

- 20x saline-sodium citrate (SSC) buffer: 100 mL
- UltraPure water: 400 mL

Store solution at Room temperature for up to a year

9 Prepare HCR amplification buffer, by combining:

- 20x saline-sodium citrate (SSC) buffer: 5 mL
- Ethylene carbonate: 5 mL
- UltraPure water: 35 mL

in a 50 mL conical tube. Store solution at Room temperature for up to 3 months.

Note

Scale up or down volumes based on needs.

Ethylene carbonate is a non-toxic substitute for formamide, and may also help to reduce background from non-specific interaction (as compared to formamide-based wash buffers). Substituting ethylene carbonate for formamide, at the same concentration, should yield qualitatively similar results.

HCR amplification buffer can also be obtained from Molecular Instruments (**https://www.molecularinstruments.com/**)

10 Prepare 1x PBS, by adding:

- 10x PBS: 55.5 mL

To a 500 mL bottle of UltraPure water.

11 Prepare heparinized PBS, by combining:

- 1 PBS packet
- Heparin sodium salt: 50 mg
- MilliQ water (or equivalent): 1 L

Shake to combine and store at Room temperature for up to 3 months.

12 (Optional) Prepare 1x PBS-Tx, by combining:

- 10x PBS: 50 mL
- 10% Triton X-100: 5 mL
- UltraPure water: 445 mL

Note

1x PBS-Tx is used if you opt to add incorporate antibody labeling to the protocol.

Sample preparation

13 General note on sample preparation and scaling

> **Note**
>
> AAV-Zombie requires that samples be fixed by non-crosslinking fixatives (methanol and acetic acid). This fixation step occurs **after** tissue is flash-frozen and sliced. Thus, planning to use AAV-Zombie for detection of AAV genomes in an animal does not preclude other analyses performed on that animal. E.g. some pieces of tissue may be processed for AAV-Zombie, some post-fixed for standard IHC, and some dissociated for single cell analyses.
>
> Make a plan for which assays you will perform on the animals, in order to maximize biological information.
>
> Note that given the cost of the reagents involved in AAV-Zombie, scaling up reactions can become costly. We recommend putting as putting as many pieces of tissue onto 1 slide as possible. Alternatively, scale down reactions and use smaller coverslips if assaying fewer tissue sections on a slide.

14 Transduce animals with AAVs carrying Zombie barcode and phage RNA polymerase promoter (barcode sequences can be found in Supplementary Table 1 of Askary et al., *Nat Biotechnol 38*: 66).

> **Note**
>
> This protocol enables spatial detection of both AAV genomes and concatemers in tissue. For detection of AAV genomes, a Zombie barcode and phage RNA polymerase promoter are required in a single AAV genome. For detection of AAV concatemers (SpECTr), 2 AAV genomes are required: one genome carries a Zombie barcode and the other carries a phage RNA polymerase promoter. Barcode sequences can be found in Supplementary Table 1 of Askary et al., *Nat Biotechnol 38*: 66.
>
> We have used this protocol to detect scAAV and ssAAV genomes and concatemers, delivered systemically to animals by AAV9, AAV-PHP.eB and AAV.CAP-B10.
>
> In choosing a phage RNA polymerase and promoter, we have relied primarily on T7. We have also used SP6 RNA polymerase successfully, but this yields smaller size spots. We have not tested T3 RNA polymerase in this application, but according to Askary et al., *Nat Biotechnol 38*: 66, this should yield similar results to T7 RNA polymerase.

15 On day of tissue harvest, prepare work area. In addition to tools and equipment for transcardial perfusion and tissue dissection, you will need:

- ice-cold 1x PBS
- clean 10 cm petri dishes
- ice
- tissue embedding molds
- O.C.T. compound
- dry ice
- dry ice-ethanol slurry

Before beginning tissue collection, label all tubes and embedding molds with solvent-resistant marker. Place a small amount of O.C.T. compound into the bottom of each embedding mold.

If analysis of fixed tissue is also desired (e.g. for detection of an AAV-encoded fluorescent protein), prepare tubes with appropriate volumes of 4% PFA in 1x PBS and keep on ice. In addition, tissue may be collected and homogenized, dissociated, or snap-frozen for other downstream analyses.

Note

Collection time is dependent upon experimental question. Because AAV-Zombie detects the AAV DNA, there is no reason to wait until reporter gene expression (e.g. GFP). However, as AAV-Zombie is compatible with RNA-based readouts of reporter gene expression, it may be beneficial to wait until detectable reporter expression to allow for paired DNA and RNA readout.

MAA fixes cells by causing proteins to precipitate, but will not crosslink molecules. Avoiding crosslinking fixatives is necessary as crosslinking will interfere with the *in situ* transcription reaction.

16 Begin tissue collection. Euthanize animals according to your lab's approved protocol, then transcardially perfuse animals with 30 mL of heparinized PBS. Working quickly, dissect out and place tissue of interest into a petri dish with ice-cold PBS, then isolate regions of interest. For AAV-Zombie, dissect tissue into small blocks (max size 0.5 cm x 0.5 cm x 0.5 cm), then place into embedding mold. Using a clean pipette tip, gently position tissue. Once the mold is full, add enough O.C.T. compound to cover the tissue, then lower the mold into the dry ice-ethanol slurry. Be careful not to let any ethanol spill into the O.C.T. Once the entire block is frozen, transfer the block to dry ice (or to a -70 °C freezer).

If also collecting tissue to fix, gently drop tissue into 4% PFA in 1x PBS, and post-fix at 4 °C, for Overnight, then proceed with standard procedures. Fresh tissue may also be harvested and homogenized, dissociated or snap-frozen for other downstream analyses.

Note

CRITICAL Do not perfuse animals with PFA, as crosslinking fixatives will interfere with *in situ* transcription of the Zombie barcode. If PFA-fixed tissue is required for separate assays (IHC, analysis of fluorescent protein expression), then separately post-fix tissue pieces for that purpose. Be careful not to contaminate samples for AAV-Zombie analysis with PFA.

Multiple tissue pieces can be put into the same embedding mold and frozen together. This can be useful if wanting to assay multiple tissues.

Rapid tissue freezing in a dry ice-ethanol slurry is necessary to mitigate disruption to tissue morphology from ice crystal formation.

17 Slice tissue. Remove flash frozen blocks of tissue from -70 °C, and allow to warm to cryostat temperature. Section tissue at 20 µm and collect sections on clean glass slides. Allow sections to dry on the top of the cryostat, then transfer to a slide box, seal with tape, and place into -70 °C storage until use.

Note

Optimal temperature for the cryostat chamber and chuck are dependent on the samples being sectioned and the desired thickness. Slightly thinner or thicker sections may be desirable for some tissues or readouts. We do not recommend using thick sections (e.g. > 50 µm), as diffusion of the enzymes that are used in AAV-Zombie into the tissue can be limited.

Label glass slides with pencil or ethanol-resistant marker. These slides will be submerged in methanol acetic acid fixation solution, which will cause most inks to wash away.

Allowing the sections to dry before placing into -70 °C helps to prevent ice crystal formation that can disrupt the tissue morphology. Allow sections to dry to the point of being transparent. Be careful not to let the tissue become too dry. If the edges of the tissue sections are becoming white, it is too dry.

We have performed AAV-Zombie on sliced sections stored at -70 °C up to 6 months, with no discernible effect on staining quality.

Tissue MAA fixation

18 Remove slide box from -70 °C storage and allow to warm to room temperature before removing tape and opening.

> **Note**
>
> Unused slides can be returned to -70 °C storage and processed at a later time.

19 Briefly wash slides by submerging them in 1x PBS, in a Coplin jar, for 00:05:00 . This step is to remove O.C.T. from the slide before fixation. Leave slides in 1x PBS until ready for fixation step. 5m

20 Fix sections in MAA. Fill a Coplin jar with ice cold MAA fixation solution. Remove slides from 1x PBS, and hold vertically to allow excess buffer to run off the slide. Touch slide to a piece of paper towel to further remove excess buffer. Submerge slides in MAA, and transfer to -20 °C freezer to fix for 03:00:00 . 3h

> **Note**
>
> MAA fixes cells by causing proteins to precipitate, but will not crosslink molecules. Avoiding crosslinking fixatives is necessary as crosslinking will interfere with the *in situ* transcription reaction.

21 Following fixation, decant MAA into an appropriate container for disposal. Fill Coplin jar with ice-cold 70% ethanol. Decant wash ethanol into MAA waste container, and fill Coplin jar again with ice-cold 70% ethanol. Transfer samples to -20 °C until processing.

> **Note**
>
> We have processed tissue samples that were stored up to 1 week in 70% ethanol at -20 °C , and achieved the expected results.
>
> Given that we have also processed cultured cell samples that were stored up to 3 months in 70% ethanol at -20 °C , and achieved the expected results, we expect that tissue sections could also be stored for longer than 1 week.

In situ transcription and probe hybridization

1h

22 General note on section

> **Note**
>
> All volumes and procedures in following section assume that the samples are tissue slices mounted on 25 mm x 75 mm glass slides. Scale up volumes appropriately if larger sized slides are used.
>
> For this section, incubations and stringent washes are performed in a staining tray (e.g. https://www.emsdiasum.com/10-slide-staining-tray-with-black-lid-each), and fixation and other wash steps are performed in Coplin jars. Washes and fixation steps can also be performed in 50 mL tubes, with 1-2 slides per tube (if processing 2 slides, ensure these are positioned back-to-back to avoid disrupting the tissue).

23 Prepare the staining tray for the *in situ* transcription reaction. Pour 100 mL of MilliQ (or comparable) water in the base of the tray to provide a humidified environment. Place the staining tray in 37 °C incubator for at least 01:00:00 . 1h

> **Note**
>
> This staining tray will serve as a humidified reaction chamber for the *in situ* transcription reaction to take place.

24 Once staining tray is prepared, thaw the NTP(s) and buffer at Room temperature , then place on ice.

> **Note**
>
> The suggested kit provides each nucleoside triphosphate (NTP) separately (i.e. ATP, UTP, CTP, GTP). To simplify reaction setup, mix together NTPs at equal volumes, then aliquot and store at -20 °C .

25 Set up *in situ* transcription reaction solution. For each slide, prepare 200 µL of reaction solution. If using T7 RNA polymerase, prepare the following reaction mix:

- NTPs: 80 µL (or 20 µL of each NTP, if storing separately)
- UltraPure water: 80 µL
- RNA polymerase: 20 µL
- Buffer: 20 µL

If using SP6 RNA polymerase, prepare the following reaction mix:

- NTPs: 80 µL (or 20 µL of each NTP, if storing separately)
- UltraPure water: 60 µL
- RNA polymerase: 40 µL
- Buffer: 20 µL

Store reaction solution on ice until use.

Note

The recommended T7 and SP6 RNA polymerase *in vitro* transcription kits use separate buffers and separate NTP concentrations. Use the buffers and NTPs specific to the polymerase used.

In our hands, multiplexing T7 and SP6 reactions in a single reaction produced very small spots from SP6-transcribed barcodes. Thus, we recommend performing separate T7 and SP6 reactions in a single reaction, use T7 buffer and T7 NTPs. However, if multiplexing both polymerases, use the buffer and NTPs for T7 RNA polymerase.

Prepare slightly more transcription solution than necessary, especially if processing many slides.

26 Set up tools and disposables. You will need:

- Kimwipes
- A pair of clean forceps (suitable for lifting and manipulating slides)
- Appropriately sized coverslips (for a 25 mm x 75 mm slide full of tissue sections, use a 24 mm x 60 mm coverslip)
- A 50 mL tube with 50 mL of UltraPure water.

27 Retrieve samples from -20 °C storage. Decant the 70% ethanol into a waste beaker, then fill the jar with 1x PBS. Decant wash then refill jar with 1x PBS and keep at Room temperature until use.

28 Start *in situ* transcription reactions. 30s

1. Remove staining tray chamber from 37 °C incubator.
2. Lift each slide out of Caplin jar, wash briefly by submerging in UltraPure water, then remove excess water from slide by leaning slide against a vertical surface for 00:00:30
3. Gently tap bottom of slide against Kimwipe or a piece of paper towel on the bench surface to remove pooled water, then dry the back of the slide with a Kimwipe. Place slide onto the staining tray, with the tissue facing up.
4. Pipette 200 µL of reaction mixture (Step 24) onto the slide
5. Spread the reaction mixture over the tissue by gently lowering a clean coverslip over the tissue, being careful not to introduce bubbles
6. Repeat for each slide

29 Transfer staining tray to 37 °C incubator, and incubate for 03:00:00 . 3h

Note

For tissue sections, especially thicker sections, it may be beneficial to initially incubate the samples with the reaction mixture at 4 °C Overnight , before transferring to a prewarmed staining tray for 03:00:00 . This may help the enzyme diffuse into the tissue while limiting transcription of the barcode (as the polymerase will be less active at 4 °C).

30 During *in situ* transcription, pre-warm the hybridization buffer to 42 °C . You will need 1.5 mL of hybridization buffer per slide.

Note

1 mL of the hybridization buffer will be used in a pre-hybridization equilibration step (Step 26) and 500 µL will be used for probe hybridization (Step 28). Prewarm extra hybridization solution.

31 Once the *in situ* transcription is complete, fix nascent transcripts in place with PFA. Fill a Coplin jar with ice-cold 4% PFA in 1x PBS. Retrieve staining tray from incubator. Pick up each slide and hold vertically, allowing the coverslip to slide off. 30m

Transfer to the 4% PFA, 1x PBS, and fix for 00:30:00 at Room temperature .

Note

Avoid pulling the coverslip off with your fingers, as this may cause damage to or smearing of the tissue. If the coverslip is not sliding off, immerse the slide and coverslip in a 50 mL tube filled with 1x PBS for 00:00:10 , then try again to remove the coverslip.

PFA will crosslink molecules in the samples, including nascent RNA from the *in situ* transcription reaction. This fixation step ensures that barcoded transcripts do not diffuse from the template DNA, enabling spatial detection of the AAV genome through the RNA proxy.

As residual PFA in the Coplin jar may interfere with other steps, dedicate a Coplin jar to PFA fixation.

32 Following fixation, transfer slides to a new Coplin jar filled with 1x PBS. Wash twice in 1x PBS to remove residual PFA, then wash twice in 5x SSC (00:05:00 per wash). 1h 5m 30s

Lift each slide out of Caplin jar and remove excess buffer by leaning slide against a vertical surface for 00:00:30 . Gently tap bottom of slide against Kimwipe or a piece of paper towel on the bench surface, then dry the back of the slide with a Kimwipe. Place slide onto the staining tray, with the tissue facing up.

Add 1 mL of pre-warmed hybridization buffer, ensuring that all the tissue is covered by hybridization buffer. Incubate at 37 °C for 01:00:00 .

33 During incubation in Step 31, prepare probe solution by diluting probes to 4 nM in the remaining hybridization buffer. Return to 42 °C until use.

Note

Probes for Zombie barcodes, AAV reporter gene transcripts and for endogenous transcripts can be pooled at this point.

Probes can be obtained as ssDNA oligos, e.g. from Integrated DNA Technologies (IDT).

We store working solutions of probes at 1 uM in -20 °C . These are prepared either by combining and diluting 100 uM stocks of each separate probe, or by obtaining a pre-pooled mixture of probes from IDT and resuspending these to 1 uM.

34 Following equilibration in hybridization buffer, remove old hybridization buffer from slides by tilting over paper towel. Remove excess hybridization buffer by leaning slide against a vertical surface for 00:00:30 . Gently tap bottom of slide against Kimwipe or a piece of paper towel on the bench surface to remove pooled hybridization buffer. 30s

Return slides to staining tray, and add 500 µL of prewarmed probe solution (Step 32). Spread the probe solution over the tissue by gently lowering a clean coverslip over the tissue, being careful not to introduce bubbles.

Incubate at 37 °C Overnight .

Note

Be careful when transporting the staining tray back to the incubator, as the coverslips may slide off. Alternatively, place staining tray in the incubator before adding probe solution and coverslipping.

Probe washes and Hybridization chain reaction (HCR) amplification 30m

35 Note on reaction volumes

Note

All volumes and procedures in following section assume that the samples are tissue slices mounted on 25 mm x 75 mm glass slides. Scale up volumes appropriately if larger sized slides are used.

For this section, stringent washes and HCR amplification is performed in a staining tray (e.g. https://www.emsdiasum.com/10-slide-staining-tray-with-black-lid-each). Other wash steps are performed in Coplin jars. Washes and fixation steps can also be performed in 50 mL tubes, with 1-2 slides per tube (if processing 2 slides, ensure these are positioned back-to-back to avoid disrupting the tissue).

36 Pre-warm stringent wash buffer to 37 °C , vortexing a couple times to ensure components are thoroughly mixed. You will need 3 mL of stringent wash buffer per slide.

37 Once stringent wash buffer is warmed to 37 °C and mixed, begin stringent washes. 31m 30s

Retrieve staining tray from incubator. Remove coverslip by holding slide vertically over a piece of paper towel, allowing the coverslip to slide off. Remove excess probe solution by leaning slide against a vertical surface for 00:00:30 . Gently tap bottom of slide against Kimwipe or a piece of paper towel on the bench surface to remove pooled hybridization buffer.

Return slides to staining tray and pipette 1 mL of stringent wash buffer onto the slide. After 00:01:00 , remove that rinse by gently lifting slide and dumping stringent wash buffer into a waste collection beaker. Return slide to staining tray, then place staining tray into the 37 °C incubator, then add 1 mL of stringent wash buffer. Place lid on staining tray and incubate for 00:30:00 .

Repeat wash step 1 more time.

Note

If the coverslips do not easily slide off, start the process by gently pushing or pulling the coverslip down the slide. Do not lift the coverslip off the slide, as this may damage the tissue.

Stringent washes may also be done in a prewarmed Coplin jar, especially if processing many slides.

Keep stringent wash buffer at 37 °C during washes.

38 Retrieve staining tray from incubator. Remove stringent wash buffer from slides by decanting slide into waste beaker, then lean slide against a vertical surface for 00:00:30 . Gently tap bottom of slide against Kimwipe or a piece of paper towel on the bench surface to remove pooled stringent wash buffer. 30m 30s

Transfer slides to a Coplin jar. Wash slides in wash buffer (5x SSC, with 0.1% Tween-20). Perform 1 brief rinse in wash buffer, followed by two longer washes at Room temperature for 00:30:00 each.

39 Following washes, remove slides from Coplin jar and lean against a vertical surface for 00:00:30 . Gently tap bottom of slide against Kimwipe or a piece of paper towel on the bench surface to remove pooled wash buffer. 30s

Dry the back of the slides with a Kimwipe, then return to staining tray. Add 1 mL of HCR amplification buffer, ensuring that the buffer distributes over the slide surface. Equilibrate

samples in HCR amplification buffer for 00:00:00 at Room temperature .

40 During HCR amplification buffer equilibration step, prepare HCR hairpins. 12m

1. Aliquot each hairpin separately into PCR tubes
2. Using a thermocycler, heat hairpins to 95 °C for 00:02:00
3. Remove hairpins from thermocycler and allow to cool back down to room temperature in the dark (at least 00:10:00

Note

Snap-cooling hairpins is critical, as it allows the hairpins to re-fold properly.

CRITICAL Do not mix hairpins together before snap-cooling, as this may cause them to hybridize to one another. Only mix the hairpins together in amplification buffer, and only once they have cooled down to room temperature.

41 Following equilibration in HCR amplification buffer, prepare HCR amplification solution. For each slide, prepare 300 µL of amplification solution. Dilute snap-cooled hairpins to 60 nM in the HCR amplification buffer.

For example, for one coverslip with one probe set using the B5 initiator, combine:

- HCR amplification buffer: 288 µL
- Hairpin 1 (e.g. B5H1): 6 µL
- Hairpin 2 (e.g. B5H2): 6 µL

Briefly vortex to mix.

Note

Prepare extra amplification solution.

42 Remove old amplification buffer from slides by tilting over a piece of paper towel or waste collection beaker. Return slides to staining tray and add 300 µL of amplification solution. Spread the HCR amplification solution over the tissue by gently lowering a clean coverslip over 4h

the tissue, being careful not to introduce bubbles. Incubate 01:00:00 to 03:00:00 at Room temperature and in the dark.

Note

From this point on, keep samples in dark as much as possible.

If only detecting Zombie transcripts, a 1 hour incubation may be preferred, to limit the size of resulting spots. However, if also detecting endogenous genes or reporter transcript, it may be necessary to do a 3 hour incubation.

Alternatively, incubate samples in amplification solution with hairpins for endogenous genes overnight, then switch to an amplification solution containing all hairpins for the final hour.

When switching between amplification solutions, it is not necessary to wash slides with other buffer. Just remove the coverslip by holding the slide vertically, then remove excess amplification solution, then add new amplification solution and a new coverslip.

43 Following HCR amplification, remove coverslips by holding the slide vertically, allowing the coverslip to slide off. 10m

Remove unassembled hairpins by washing 4 times slides in wash buffer (5x SSC, with 0.1% Tween-20), in a Coplin jar. Perform 1 brief rinse with wash buffer, followed by three longer washes at Room temperature for 00:10:00 each.

44 (Optional) Label proteins with immunofluorescence staining. 1h 15m

We expect that most standard immunofluorescence protocols can be used here. Below is the protocol we used for labeling AAV capsid proteins with immunofluorescence using a fluorophore-conjugated primary antibody. Optimal incubation conditions, dilutions, and durations, will need to be determined empirically for each protocol and antibody.

1. Block slides in 1 mL of BlockAid blocking buffer with 0.1% Triton X-100, for 01:00:00 at Room temperature
2. Incubate samples with 1:100 dilution of primary antibody in BlockAid blocking buffer with 0.1% Triton X-100, 4 °C for Overnight .
3. Wash samples 3 times in 1x PBS-Tx, for 00:15:00 each at Room temperature

Note

We successfully detected capsid proteins with immunofluorescence staining via a fluorophore-conjugated monoclonal antibody against linear capsid epitopes (clone B1). As MAA fixation is expected to denature proteins, we suggest trying antibodies against linear epitopes vs. those against conformational epitopes.

45 (Optional) Label nucleus with Hoescht 33342. 35m

For each slide, prepare 1 mL of 1x PBS with 1/10000 Hoechst 33342. Rinse slides briefly in 1x PBS in a Coplin jar, then return to staining tray. Pipette 1 mL of Hoechst solution directly onto slide and incubate for 00:30:00 at Room temperature in the dark.

Following incubation, wash samples 3 times with 1 mL of 1x PBS at Room temperature for 00:05:00 each.

Note

DAPI can be used in place of Hoechst, with no changes to the protocol.

46 Mount coverslips onto slides. You will need a 50 mL tube with 50 mL of UltraPure water, a suitable mounting medium, and appropriately-sized glass coverslips.

For each slide:

1. Remove slide from Coplin jar, and immerse briefly in UltraPure water
2. Transfer slides to a fume hood, allowing the tissue to dry to the point of being transparent
3. Once tissue is dry, pipette 100 µL of mounting media in a line on the surface of the slide, near the tissue sections
4. Spread the mounting media over the tissue by gently lowering a clean coverslip over the tissue, being careful not to introduce bubbles.

Once all coverslips are mounted, gently press down on each coverslip to push out excess mounting media. Store in the dark at Room temperature for at least Overnight until mounting media cures. Once cured, excess Prolong Diamond can be easily removed by running slides under a gentle stream of MilliQ water, being careful to not displace coverslips.

Samples are now ready to image.

Note

Be careful not to dry tissue too long. If the edges of the tissue are turning white, the tissue is becoming too dry.

For mounting coverslips, we use Prolong Diamond, but any mounting medium compatible with fluorescence signal should work. If using another mounting medium, follow manufacturer directions for that mounting medium.

Protocol references

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