TDP-43 RNA Aptamer IHC Staining

Title

TDP-43 RNA aptamer staining to detect pathological TDP-43 in FFPE human tissue, as described in Spence and Waldron et al., 2024 (Acta Neuropathologica): A SOP and tick-sheet. v2.
Fergal M. Waldron*, Holly Spence*, Jenna M. Gregory‡
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*equal contributions, ‡corresponding author.

Purpose

The purpose of this SOP is to outline the correct procedures for performing Immunohistochemistry (IHC) to detect pathological TDP-43 in FFPE-preserved human tissue using the TDP-43 RNA Aptamer (TDP-43^{APT}), as described in Spence and Waldron *et al*., 2024 published in *Acta Neuropathologica* (see citation below).

This protocol uses the TDP-43^{APT} published in Zacco *et al*., 2022. The sequence is: CGGUGUUGCU with a 3' Biotin-TEG modification, purified using HPLC, scale: 1.0 µM synthesis.

Protocol timescale

Users with access to Sequenza immunostaining racks and histological facilities (with fume hood) should be able to carry out all steps over 2 days. These can be summarised as:

- Day 1: First day of TDP-43^{Apt} staining (with essential overnight fixation step in Formaldehyde/PFA).
- Day 2: Second day of TDP-43^{Apt} staining with Anti-Biotin (HRP) secondary antibody, and DAB chromogen.

References for citation of this method

Please cite both of these if using this method:

The citation, Spence and Waldron *et al*., 2024, for the first publication for the development, modification and employment of the TDP-43 RNA aptamer to stain human tissue published in *Acta Neuropathologica*.

> **RNA aptamer reveals nuclear TDP-43 pathology is an early aggregation event that coincides with STMN-2 cryptic splicing and precedes clinical manifestation in ALS.**
> Holly Spence*, Fergal M. Waldron*, Rebecca S. Saleeb, Anna-Leigh Brown, Olivia M. Rifai, Martina Gilodi, Fiona Read, Kristine Roberts, Gillian Milne, Debbie Wilkinson, Judi O'Shaughnessy, Annalisa Pastore, Pietro Fratta, Neil Shneider, Gian Gaetano Tartaglia, Elsa Zacco, Mathew H. Horrocks, Jenna M. Gregory‡ (2024).
> *Acta Neuropathologica* 2024 Mar 5;147(1):50. DOI: 10.1007/s00401-024-02705-1.
> *equal contributions, ‡corresponding author.

The citation, Waldron and Spence *et al*., 2024, for this SOP published on *protocols.io* is:

> **TDP-43 RNA aptamer staining to detect pathological TDP-43 in FFPE human tissue, as described in Spence and Waldron et al., 2024 (Acta Neuropathologica): A SOP and tick-sheet. v2.**
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Safety First

Before starting, please seek out and read all relevant Health & Safety documentation, and fully read this SOP. Before working please make sure these have been carried out.

- ✓ COSHH assessment
- ✓ Risk assessment
- ✓ Safe System of Work
- ✓ SOP read and understood

Special note

See Appendices for materials and solutions.

Day 1 (of 2)

- First day of TDP-43^{Apt} staining (with essential overnight fixation step in Formaldehyde/PFA).

1. Prepare treatment materials

- ✓ Peroxidase block and Avidin/Biotin blocking reagents from cool storage (4°C), bring to room temperature.
- ✓ Make up 1x TBS
- ✓ If using, make up citric acid buffer (see step 3).
- ✓ Get Sequenza slide racks and coverplates.

2. Deparaffinise FFPE sections and remove formalin pigment

2a: Deparaffinise FFPE slides	
Xylene	**3 min**
Xylene	**3 min**
Alcohol	**2 min**
Alcohol	**2 min**
Place slides in tap water (but see below)	
NB: Do not place slides in tap water if employing the optional Picric Acid step below	

2b: Optional remove formalin pigment	
(Optional step) Saturated Alcoholic Picric acid	**15 min**
Wash in warm, running tap water	**15 min**

3. Antigen/Epitope retrieval

The pre-treatment most often used in The Gregory Lab is heat induced epitope retrieval with 10 mM citric acid (pH 6 – NB: pH must be within 0.1 of 6), you will need to make up a working solution.

☞ For 10mM (working) solution dilute 100mM stock solution 1:10 with dH_2O.
e.g.To make up 500 ml citric acid buffer solution sufficient to fill a typical histology staining jar, dish or tub.
- 50ml 100mM citric acid stock solution and 450ml dH_2O.

Using the pressure cooker for antigen/epitope retrieval

Antigen retrieval can be carried out us a commercially available household pressure cooker – here, the "3L Drew and Cole Pressure King Pro".

For first time users and/or a more detailed step-by-step description see Appendix C, "Using the pressure cooker: Step-by-step".

Alternatively see section, "Using the pressure cooker: Summary" below.

Using the pressure cooker: Summary

The settings, times and temperature on your pressure cooker may be different to the one used in the Gregory Lab. The instructions below are optimised for a 3L Drew and Cole 'Pressure King Pro' domestic pressure cooker.

Put **500ml dH_2O** in the basin of the pressure cooker.

Place slides in a slide rack, place the rack in a suitable, pressure cooker compatible, container (e.g. histological slide staining jar, dish or tub) and pour in antigen retrieval buffer (10mM citric acid at pH 6). Make sure the slides are fully emersed in the antigen retrieval buffer.
Place container in pressure cooker.
Put the lid on and lock it by turning it anticlockwise until it clicks.
Turn the pressure release valve to the closed position.

Press the 'browning/meat' button and use the + and – buttons to adjust the time to **15 min** (if it starts before you're ready, press cancel and try again). After a short delay, the pressure cooker will start automatically. The full cycle takes around **20 min** (including warming up and cooling time).
When the cycle has finished there will be a short beep and the display will read 'end', press the cancel button. You can wait for the pressure to release naturally, or you can move the valve to the open position to release the pressure more quickly.

Beware! The slides and pressure cooker will be very hot and should be handled with extreme caution.
Remove the lid of the pressure cooker, lift the basin out and place it in the sink.
Cool the slides down in running tap water.

4. Coverplating

These steps are for users that have access to Sequenza racks for immunostaining. This protocol is optimised for Sequenza rack set up – please adjust reagent quantities accordingly if you are not using this set-up.
Use **dH_2O** to fit coverplates to your slides.
Make sure there are no bubbles present and press securely into the Sequenza slide rack.
Fill the wells to the top with **dH_2O**, leave for **5 min.**

5. Blocking

Apply the peroxidase block to block endogenous peroxidases in tissue. We use 3% H_2O_2 (peroxidase blocking solution supplied with the Leica Novolink Polymer Detection kit). Avidin/Biotin blocking will also be required, we use the Abcam kit for this dH_2O (see appendix for details). See below for how to make up working TDP-43^{APT}, 4%

formaldehyde, anti-biotin secondary antibody and DAB solutions. All reagents and solutions (other than TDP-43^{APT} itself which should be stored in aliquots at -80°C and defrosted on ice for use) must be brought up to room temperature for use.

Day 1 Procedure including antigen retrieval, blocking & overnight fixation

Antigen retrieval (citric acid pH6)	**15 min**
Coverplate	
dH_2O	**5 min**
Peroxidase block (3 drops)	**30 min**
Wash with TBS	**5 min**
Avidin block (4 drops)	**15 min**
Wash with TBS	**5 min**
Biotin block (4 drops)	**15 min**
Wash with TBS	**5 min**
Prepare aptamer (1:500 dH_20)	**5 min**
Wash with dH_20	**5 min**
Aptamer incubation	**3 hrs (4°C, dark)**
Fix step 4% PFA in dH_20	Overnight (4°C, dark)

…… Stopping point: End of day 1 (of 2) ……

Day 2 (of 3)

7. Anti-Biotin staining with Fast Red Chromogen

- Second day of TDP-43^{Apt} staining with Anti-Biotin (HRP.) secondary antibody, and DAB chromogen.

See below for how to make up working anti-biotin secondary antibody and DAB solutions. All reagents and solutions (other than TDP-43^{APT} itself which should be stored in aliquots at -80°C and defrosted on ice for use) must be brought up to room temperature for use.

7.1 Procedure: Anti-biotin staining with Fast Red Chromogen

Re-coverplate to Remove bubbles	**Optional**
Wash with dH_20	**5 min**
Anti-biotin/HRP 1:100 in MilliQ H20	30 min
Wash with dH_20	**5 min**
DAB (1:20)	**5 min**
Wash with dH_20	**5 min**

Making up reagents and solution

When making up reagents/solutions we need to know
- What is the final volume required for my samples?
- What is the appropriate dilution for my reagents/solutions?
- How much reagent/solution will be needed?

- Each slide requires 100µl of solution (for Sequenza racks)
  - Therefore, number of slides x 100µl = volume of solution required. So if we have 10 slides we will require 1000µl of solution (10 x 100 = 1000)

Making up TDP-43 RNA Aptamer (TDP-43^{APT}) for use

TDP-43^{APT} is an RNA aptamer and should therefore be stored at -80°C and defrosted on ice for use. TDP-43^{APT} is biotinylated and is therefore light-sensitive so should be prepared and used with minimum exposure to light. Upon receipt of the TDP-43^{APT} it is recommended to prepare small aliquots for use to minimise the number of pre-use freeze-thaw cycles as it is an RNA molecule.

For use, defrost TDP-43^{APT} on ice (in dark), and dilute 1:500 in MilliQ water.

Formaldehyde (4% working solution)

Our formaldehyde comes in 1 ml glass ampules at 16% (formaldehyde, 16% w/v aq. Soln., methanol free) and is stored at room temperature. Make 4 ml of 4% formaldehyde by mixing 1 ml of 16% PFA with 3 ml of dH_2O.

DAB

DAB chromogen begins to precipitate soon after being diluted in substrate buffer so prepare DAB solution immediately (i.e., < 2 mins) before use. DAB chromogen is light sensitive so prepare and use with minimum exposure to light)

The ratio of DAB chromogen to DAB substrate buffer is 1:20 Making 50µl:1000µl is plenty of solution for 10 slides. Pipette **100µl** of DAB solution into the well of each coverplate, leave for **5 min.**

Fill up wells with **dH_2O**, leave for **5 min**

Anti-Biotin (Horseradish Peroxidase) Secondary Antibody

Anti-Biotin/HRP Stored at 4°C but brought up to room temperature and diluted 1:100 in dH_2O for use.

Avidin/Biotin Blocking

We use Avidin/Biotin Blocking kits which we store at 4°C and bring up to room temperature for use.

6. Remove coverplates

Carefully remove each slide with their attached cover plate from the Sequenza slide rack, and then gently lift the slide off the coverplate (remembering to lift rather than slide them off).
Put the slides in a slide rack and wash well in running tap water for **5 min**.

7. Counterstain

Haematoxylin	**2 min**
Wash in running tap water	**1 min**
Lithium carbonate ("blueing")	**30 sec**
Wash in running tap water	**1 min**

8. Dehydrate, clear and mount/coverslip

Summary

Dry off the handle of the slide rack	
Series of alcohols (dehydrate)	Up to **2 min** in each alcohol or slides are clear
Dry off the handle of the slide rack	
Xylenes (clear)	Up to **2 min** in each xylene or slides are clear

Your slides are now ready to be coverslipped.

…….. Stopping point: End of day 2 (of 2) ……

Appendix A

Materials

Biological materials

- FFPE tissue slides

Reagents

Histology

- Xylene
- Ethanol
- Haematoxylin
- Lithium carbonate
- DPX mountant (or similar)

Immunohistochemistry

- Citric acid
- 5N Hydrochloric acid & 5N Sodium hydroxide to bring citric acid to pH 6
- MilliQ H_2O
- Distilled H_20
- Peroxidase block (3% H_2O_2)
- TBS
- Avidin/Biotin Block: e.g. Avidin/Biotin Blocking Kit (Abcam, ab64212)
- TDP-43 Aptamer (TDP-43^{APT})
- 4% w/v Formaldehyde: from e.g. Pierce™ 16% Formaldehyde (w/v), Methanol-free (ThermoFisher Scientific, Cat No. 28906
- Anti-biotin (HRP) secondary antibody

Equipment

Histology

- Fume hood (for use with Xylene)
- Slides and coverslips
- Histology staining rack
- Coplan jars or similar

Immunohistochemistry

- pH meter (to make citric acid up to pH 6)
- Sequenza rack
- Sequenza coverplates
- Pressure cooker (for antigen retrieval): e.g. Drew and Cole Pressure King Pro
- Refrigerator
- Plastics and other (Pipettes and tips, Falcon tube, dropper, eppendorfs)

Appendix B

Solutions	Use	Method		Storage
Citric acid stock (check which citric acid first)	Antigen retrieval 100mM citric acid buffer	dH_2O Citric acid anhydrous Adjust to pH6	1L 19.21g	4^0C (cold room) Dilute 1:10 for working solution
	Antigen retrieval 100mM citric acid buffer	dH_2O Citric acid monohydrate Adjust to pH6	1L 21.01g	4^0C (cold room) Dilute 1:10 for working solution
5N Hydrochloric acid	pH buffer	dH_2O HCL	115ml 85ml	Acids cupboard
5N Sodium hydroxide	pH buffer	dH_2O NaOH	500ml 135g	Cupboard
TBS (Tris Buffered Saline)	Wash	Tris buffer Saline Solution	100ml 900ml	Make fresh
Tris buffer	x10 stock solution	Saline solution Tris Adjust pH to 7.6. Then make up to 1.5L with dH_2O. Check pH, adjust if necessary.	500ml 1.21g	4^0C (cold room)
Saline solution	For Tris and TBS	dH_2O NaCl	5L 42.5g	Cupboard
Saturated alcoholic picric acid	Removing formalin pigment	Add picric acid to 100% IMS until no more powder can be dissolved. Filter before use.		

Appendix C

Using the 3L Drew and Cole Pressure King Pro pressure cooker: Step-by-step

Preparation:

1. Make up antigen retrieval buffer working solution (see Appendix A)
    - 10mM citric acid adjusted to pH –> store at 4°C.

2. Put 500ml dH_2O in the orange basin of the pressure cooker.

3. Place slides in a fresh/dry histology staining rack (e.g., metal rack without handles)
    - Slides will be in tap water if coming from deparaffinisation step.

4. Place rack in histology staining cup/tub rack (this will stand in the in the cooker basin) …

5. … and pour in antigen retrieval buffer (**e.g., citric acid as in 1 above, or tris/EDTA**)
    - Make sure the slides are fully covered.

6. Place tub upright in the water in orange basin of the pressure cooker.

Using the pressure cooker:

7. Put the lid on and lock it by turning it anticlockwise until you hear a click.
    - Silver arrow on lid aligned with arrow on cooker.

8. Turn the pressure release valve to the closed position.
    - This is the dotted O position.

9. Press the 'browning/meat' button and use the + and – buttons to adjust the time to 15 min (if it starts before you're ready, press cancel and try again).

10. After a short delay, the pressure cooker will start automatically. The full cycle takes around 20 min.

11. When the cycle has finished there will be a short beep sound and the display will read 'end', press the cancel button.

12. To release the pressure, carefully (beware of steam) move the valve to the open position (or wait for the pressure to release naturally).
    - Beware! The slides and pressure cooker will be very hot and should be handled with extreme caution.

13. Remove the lid of the pressure cooker.

14. Cool the slides down in running tap water/ take slides out with tweezers and place into fresh rack in cool tap water.