TDP-43 RNA Aptamer (TDP-43^{APT}) Immunohistochemistry (IHC)

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).

Users with access to Sequenza immunostaining racks and histological facilities (with fume hood) should be able to carry out all steps over two days.

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.

Reference for citations of this method

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* (in press at the time of release of this SOP on 01/03/2024)
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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 Appendix for materials and solutions.

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**
Do not wash in water	

2b: Remove formalin pigment

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

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.

Step	Time
Day 1	
Peroxidase block (3 drops)	**30 min**
TBS	**5 min**
Avidin block (3 drops)	**15 min**
TBS	**5 min**
Biotin block (3 drops)	**15 min**
TBS	**5 min**
dH_2O	**5 min**
TDP-43^{APT} (1:500 in MilliQ H_2O)	**3 hrs @ 4°C in dark**
Essential fix step with 4% Formaldehye	**Overnight @ 4°C in dark**
Day 2	
Optional re-coverplate to remove bubbles	
dH_2O	**5 min**
Anti-Biotin (HRP) (1:100 in MilliQ H_2O)	**30 min**
dH_2O	**5 min**
DAB (1:20 chromogen to buffer)	5 min covered (light sensitive)
dH_2O	**5 mins**

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**
Alcohol (dehydrate)	Up to **2 min** in each of at least 2x 100% alcohols or until slides are clear
Xylene	Up to **2 min** in each of at least 2x xylene or until slides are clear
Mount/coverslip	

Appendix

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)

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.		