MmuPV1 L1- cytokeratin dual immunofluorescence using home-based tyramide signal amplification

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Abstract

To accomplish MmuPV L1-K14 double fluorescent staining we made use of tyramide-based signal amplification (TSA) technique. This technique allows for easy multiplexing of immunofluorescent staining but perhaps its biggest advantage is that it permits double immunofluorescent staining using two unconjugated primary antisera raised in the same species. We have adopted a home-based method of TSA staining using reagents as described previously for performing L1-K14 dual fluorescent staining. The general principle of TSA staining is described in Fig. 1. The first step involves staining of viral capsid using rabbit sera raised against L1 followed by HRP conjugated secondary antibody. The HRP-conjugated 2° antibody is then biotinylated using biotin conjugated with tyramide. We can then proceed to stain with antibody against cytokeratin-14 (raised in same species as L1 antibody, i.e. rabbit). Both antibodies can now be detected easily – i.e. L1 can be detected with anti-Streptavidin and K14 can be detected with an anti-rabbit 2° antibody. Several commercial kits also exist for performing TSA-based staining (e.g. PerkinElmer, Pierce and Thermo Scientific) and are being used readily in several laboratories.

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Guidelines

This is a home-based TSA staining protocol. Users may find it easier to purchase commercially available alternatives available from several companies such as Perkin Elmer, Fisher Scientific etc.

We recommend that when performing TSA staining, users should have the following controls:

1. Negative control - i.e. no primary antibody

2. Unanmplified control - i.e. no TSA is added to amplify signal.

Materials

TSA Blocking Buffer FP1012 by Perkin Elmer

Antigen Retrieval Buffer (pH=9) ab93684 by Abcam

Biotin-NHS H1759 by Sigma Aldrich

Tyramine Hydrochloride T2879 by Sigma Aldrich

Rabbit Sera against L1 View by Dr. Chris Buck, NIH

Keratin 14 polyclonal antibody 905301 by BioLegend

Goat-anti-rabbit-Alexafluor 488 A11008 by Thermo Fisher Scientific

Streptavidin-Alexafluor-594 conjugate S32356 by Thermo Fisher Scientific

Triethylamine 25108 by Thermo Fisher Scientific

Imidazole I5513 by Sigma

Protocol

Pretreatment of formalin-fixed tissue sections

Step 1.

1. Deparaffinize section in 3 changes of Xylene for 2 minutes each in coplin jars.
2. Rehydrate deparaffinized sections in an ethanol gradient series as follows: Twice in 100% ethanol, then once in 95%, 70%, 50%, 25%, 12.5% and final rehydration in double distilled water.
3. Wash sections twice in 1X PBS.
4. Block endogenous biotin by incubating sections in 0.3% Hydrogen peroxide in Methanol for 10 mins.
5. Wash sections twice in 1X PBS.
6. Before performing antigen retrieval, prepare a boiling water bath by placing a slide chamber filled with antigen retrieval buffer (pH=9) (abcam ab93684) in a tupperware container filled with water, and pre-heat for 7 minutes 30 seconds at highest power level in the microwave specification.
7. Place slides from Step v into the pre-heated slide chamber.
8. Microwave the slide chamber/tupperware container for 1 minute at high power to bring slides to boiling temperature.
9. Microwave for an additional 19 minutes at 30% power level.
10. Cool slides to room temperature by placing the entire slide chamber on the lab bench for at least 1.5 hours.
11. Wash slides twice in 1X PBS and then circle tissue with hydrophobic pen.
12. Wash tissue in 1X PBST (2l PBS+1gm Tween-20) 3 times for 3 minutes each

Incubation of L1 primary antibody

Step 2.

1. Apply blocking reagent (TSA blocking buffer) to tissue and incubate at room temperature for 1 hr by placing slides in a humidifier chamber. The goal is to cover the tissue section completely. For example, for a single tissue section 20µl blocking reagent is sufficient.
2. Rinse tissue in 1X PBST (2l PBS+1gm Tween-20) and apply primary antibody at desired dilution (i.e. for rabbit-anti-L1 - 1:5000 dilution). Incubate overnight in a humidified chamber at 4°C.

Preparation of biotin-tyramide stock reagent

Step 3.

1. Stock solution A (active ester) is generated by dissolving Biotin-NHS in N,N-Dimethylformamide (DMF) at 10mg/ml. This solution should be prepared freshly prior to use.
2. Stock solution B (tyramine stock) is generated by dissolving Tyramine hydrochloride in DMF at 10mg/ml. To this add 1.25-fold equivalent amount of triethylamine (10 µl of 7.2M) per ml.
3. Now add Stock A (1.1 fold equivalent amount) to the Stock solution B and leave at room temperature (RT) in the dark for 2 hrs to generate biotin-tyramide.
4. Dilute biotin-tyramide conjugates with ethanol to obtain Biotin-tyramide stock solution of 1 mg/ml.

NOTES

Aayushi Uberoi 02 Aug 2017

This method of generating biotin-tyramide has been adopted from methods described previously.

Hopman AH, Ramaekers FC, Speel EJ. 1998. Rapid synthesis of biotin-, digoxigenin-, trinitrophenyl-, and fluorochrome-labeled tyramides and their application for In situ hybridization using CARD amplification. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society **46:**771-777.

Tyramide Signal Amplification of L1

Step 4.

1. Wash slides that had been treated with anti-L1 in 1X PBST 3 times for 3 minutes each.
2. Apply Goat-anti-rabbit- HRP secondary antibody (for this example at a 1:500 dilution) in blocking buffer for 1 hr at room in a humidified chamber.
3. Wash tissue in 1X PBST (2l PBS+1gm Tween-20) 3 times for 3 minutes each.
4. During the time the tissue is being incubated with the 2^{o} antibody, prepare reaction buffer by adding 1µl 30% Hydrogen peroxide solution to 30ml 0.1M Imidazole solution.
5. Bring the biotin-tyramide stock solution (1mg/ml) to room temperature.
6. 10 minutes prior to completion of the secondary ab incubation dilute biotin-tyramide stock solution 1:100 with reaction buffer i.e. 30µl of biotin-tyramide is added to 3ml reaction buffer and incubated at room temperature.

It is critical that this sample be allowed to sit for 10 minutes.

7. Incubate slides with the biotin-tyramide-complex (from step 6 of this section) for 10mins at room temperature.

It is critical that this step be performed for 10 minutes

8. Wash tissue in 1X PBST (2l PBS+1gm Tween-20) 3 times for 3 minutes each.

K14 staining

Step 5.

1. Tissue is rinsed with 1X PBST (2l PBS+1gm Tween-20) and apply primary antibody anti-K14 (Covance) primary @ 1:1000 at room temperature for 1 hour.

Detection of L1-K14

Step 6.

1. Wash slides in 1X PBST 3 times for 3 minutes each
2. Incubate tissues with secondary detection reagents as follows: 1:200 anti-rabbit conjugated with Alexafluor488 (for K14), 1:200 anti-streptavidin conjugated with Alexafluor594 (for biotinylated L1) for 1 hour at room temperature.
3. Stain with Hoechst for 10 mins
4. Wash in PBS and coverslip with mounting media