Application Note

Antibody validation with mRNA transfection into human pluripotent stem cells

Introduction

Elixirgen Scientific's proprietary transcription factor-based technology allows rapid and reproducible differentiation of human induced pluripotent stem cells (iPSCs) or embryonic stem cells (ESCs) into neurons without sacrificing the purity of the cells[1]. Immunocytochemistry (ICC) is used as one of the tools to characterize iPSC-derived neuron cells. Although antibodies are one of the most commonly used tools in both basic and clinical research, there are no universally accepted standardized methods to validate reagents. Three key aspects are involved in antibody validation: specificity, affinity, and reproducibility. This application note describes an easy method to validate antibody specificity for immunocytochemistry using iPSCs.

Materials and methods

Cell culture and mRNA transfection

Human iPSCs (CW50065, CIRM iPSC repository) were maintained with our standard protocols[2]. For each antibody iPSCs were seeded at 25,000 viable cells per well of a 96-well plate (36 wells) on day 0 and allowed to grow until the next day. Half of the wells (18 wells) were then transfected with the mRNA encoding the target protein, either ALDH1L1 or GFAP, using Lipofectamine™ MessengerMAX™ (ThermoFisher) as a positive control. The remainder of the wells were left untransfected as a negative control. On day 2, the cells were fixed and stained with antibodies specific for the target proteins (Figure 1).

Figure 1: Schematic of the workflow for antibody validation.

Antibodies

Antibodies specific for GFAP and ALDH1L1 were used for validation. This method can be extended to other antibodies that are not expressed in iPSCs. For visualization of primary antibodies the secondary antibody Alexa Fluor 594 (1:500) was used.

Immunocytochemistry

After fixing the cells with 4% paraformaldehyde, cells were blocked with a blocking buffer. The cells transfected with mRNA were treated with three dilutions of antibodies (1:100, 1:200 and 1:500 for the GFAP antibody; 1:500, 1:1000, and 1:2000 for the ALDH1L1 antibody) and three different dilutions of IgG corresponding to the concentration of primary antibodies. Untransfected cells were treated with the same conditions. Samples were prepared in triplicate. Primary antibody incubation was done in a blocking buffer overnight at 4°C degrees. After 3 PBS washes, cells were incubated in secondary antibodies and nuclei were fluorescently stained with Hoechst 33342.

Image acquisition and analysis

To capture immunofluorescence of a single well of a 96 well plate, 4 fields were imaged with a 10x objective using an automated high-content confocal microscopy CQ1 (Yokogawa). The nucleus and antibody positive areas were segmented by an image analysis software (Cell PathFinder, Yokogawa), and the number of total cells and of target antibody positive cells were counted from the number of nuclei segmented and from the mean intensity of target antibody fluorescence in segmented area, respectively.

Results

In order to validate antibody specificity, iPSCs transfected and untransfected with target mRNA were treated with target antibodies or Immunoglobulin G (IgG) as a negative control (Figure 2A and 2B). Both transfected cells treated with ALDH1L1 and those treated with GFAP antibodies showed mosaic-like staining as the transfection efficiency is not 100%. No signal was observed in untransfected cells treated with the target antibody thus indicating no non-specific binding of the antibody. Transfected cells treated with IgG also showed no fluorescence indicating that this antibody is specific.
Captured images were analyzed by CellPathfinder. The program identified stained nucleus and cytoplasmic areas and quantified the mean intensity of fluorescence. Figure 3A shows images of transfected and

untransfected cells treated with ALDH1L1 antibody and the resulting identification/segmentation. Individual cells can be segmented by specifying nuclear and cell regions. The total cell number was counted. More than 10,000 cells were counted per well and the mean intensity of fluorescence was plotted on the histogram (Figure 3B). The IgG treatment resulted in no fluorescence.

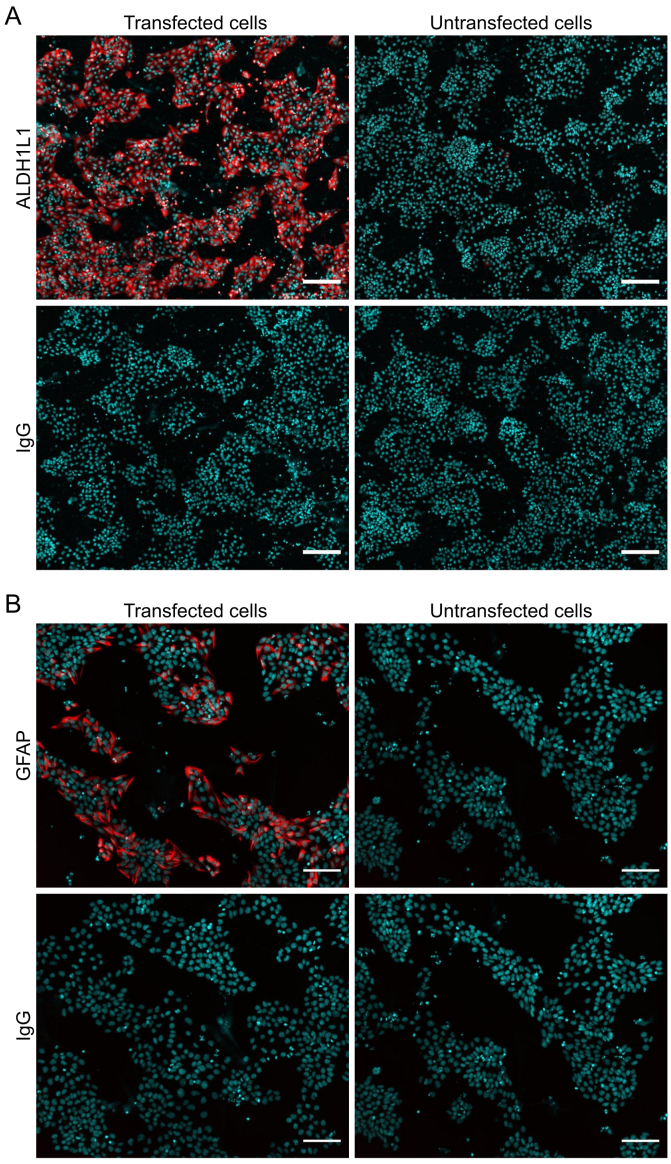

Figure 2: Fluorescence images of iPSCs treated with ALDH1L1 (A) and GFAP (B) on day 2. Blue: Hoechst 33342, Red: ALDH1L1 or GFAP. Scale bar: 100µm.

Although it was not obvious in the images, some background staining was detected with the ALDH1L1 antibody in untransfected iPS cells by CellPathfinder. The profile of transfected cells treated with ALDH1L1 showed two peaks. When a threshold was set based on untransfected cells treated with ALDH1L1, it was positioned in a valley and showed 72% positive cells in transfected cells with ALDH1L1 while the rest of the samples showed 0.1% positive cells. We categorized this antibody as "Fair". With GFAP, peaks of all negative controls indicated that there was no non-specific binding or background fluorescence in untransfected cells. A threshold was set just after the peaks of all negative controls and transfected cells treated with GFAP showed about 66% positive cells. We categorized this antibody as "Good". A dilution of primary antibody that showed minimum background and non-specific binding was chosen for further use. If any of the negative controls show more than 5% positive cells, we categorize the antibody as "Bad" and will not use it further.

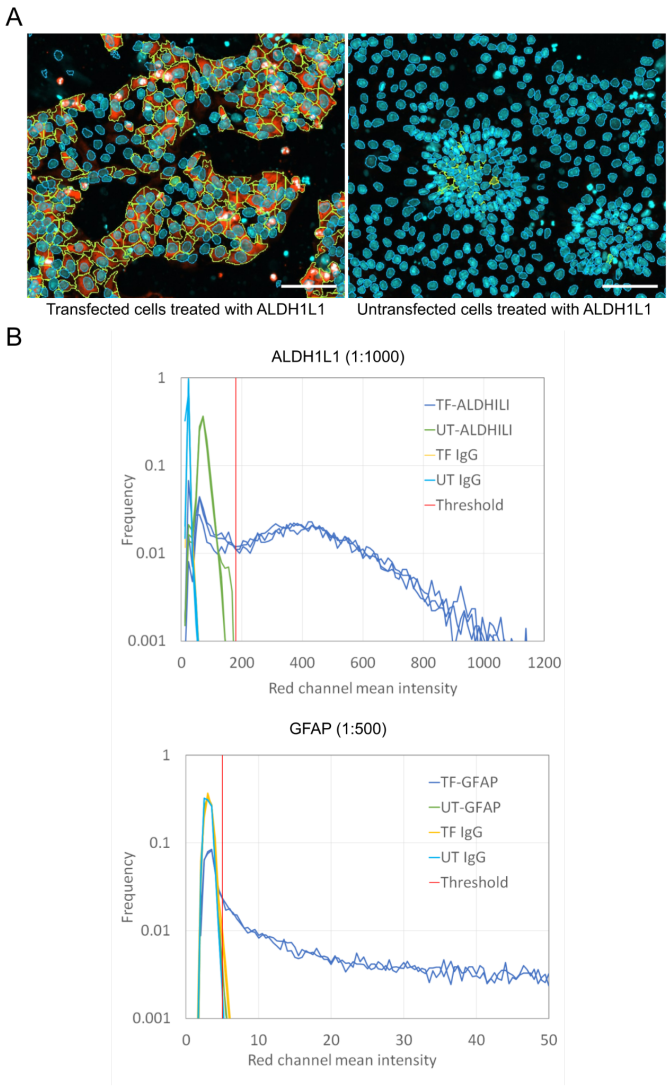

Figure 3: Nucleus area and red color positive area segmented by an image analysis software, Cell Pathfinder (A). Light blue circles mean nuclei area and yellow circles mean red fluorescent positive area. Scale bar means 100µm.
Histograms of mean red intensity in the nucleus area detected by the image analysis (B). If both negative control samples, untransfected cells stained with the target antibody and transfected cells stained with IgG, it means that the target antibody has very low non-specific binding.

Using the validated antibodies in this study, Quick-Glia™ Astrocytes - Human iPSC-derived astrocytes (AS-SeV-CW50065-S, Elixirgen Scientific) were evaluated. The astrocytes were fixed at day 43 from iPSCs and stained by GFAP and ALDH1L1 antibodies. Figure 4 shows representative images of ICC. Using IgG as a negative control, we determined the threshold for GFAP positive cells. On the other hand, as the antibody validation result suggested that ALDH1L1 had a unspecific binding, the threshold was not determined from IgG negative control result, but from a profile of histogram of ALDH1L1 fluorescent intensity. As a result, the percentage of GFAP positive cells and that of ALDH1L1 were 50% and 94%, respectively.

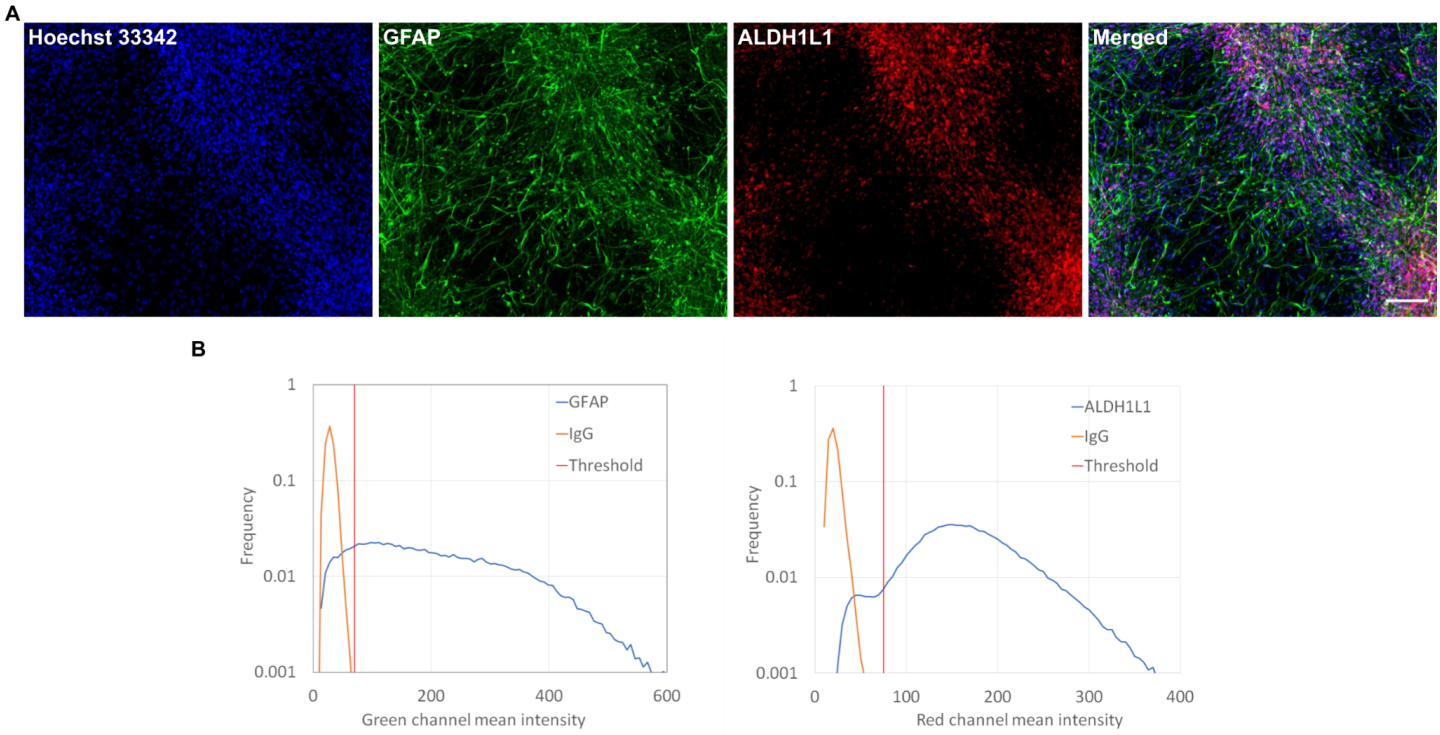

Figure 4: Fluorescence images of iPSC-derived astrocytes stained with astrocyte markers GFAP and ALDH1L1 (A) Nuclei were stained with Hoechst 33342.. Scale bar = 100μm. (B) The histograms of mean green and red intensity in the nucleus area were calculated. From the antibody validation experiment, the threshold of GFAP positive cells can be determined from IgG negative control. On the other hand, as the ALDH1L1 antibody showed some unspecific signal, the threshold was determined from the profile of the histogram.

Discussion and Summary

The variability of antibody quality has made validation of antibodies crucial. We have described a method to validate antibody specificity by transfecting iPSC with mRNA for the antibody targets. Using the CQ1 for analysis allows this method to provide quantitative data that can be used to set thresholds to categorize antibodies and determine whether they should be used in future studies.

References

1. Goparaju, S. K. et al. Rapid differentiation of human pluripotent stem cells into functional neurons by mRNAs encoding transcription factors. Sci. Rep. 7, 1–12 (2017).
2. Application protocol of Elixirgen Scientific, Maintenance of hPSC culture suitable for Quick-Tissue™ Differentiation Kits