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UTILIZING CRISPR FOR EARLY GENE TAGGING IN hiPS CELLS

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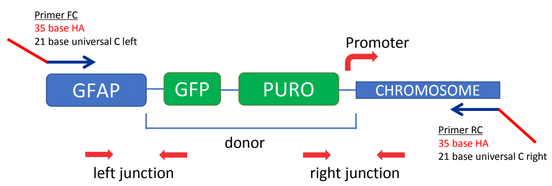
INTRODUCTION

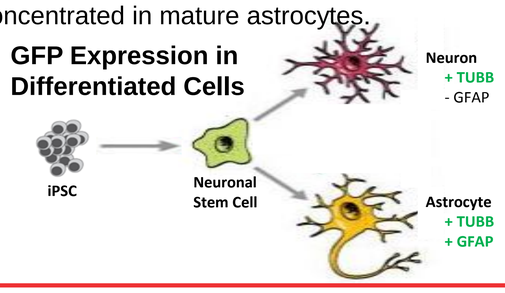
Methods to observe the differentiation of iPS cells to a desired lineage are often complex and time-consuming. To streamline this process, we developed methods to add fluorescent tags to genes that are unexpressed in the iPSC state, but expressed in the desired differentiated state. The expression of the marker after differentiation allows for rapid visual screening, minimizing the time and labor associated with clonal isolation of properly differentiated cells, and optimization of factors relevant to the differentiation process.

BACKGROUND

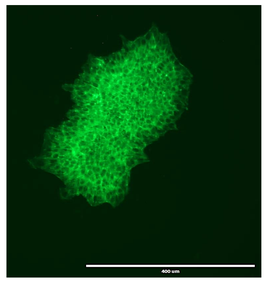
Invitrogen TrueTag™ is a platform which allows for rapid production of transfection-ready donor DNA. This illustration shows the construction of the donor:



Two genes, βIII-tubulin (TUBB) and glial fibrillary acidic protein (GFAP), were tagged in the C-terminus with GFP. Silent in iPSC's, TUBB is a protein present in neurons, while GFAP is highly concentrated in mature astrocytes.



TAGGING ACTIVE GENE IN iPSC

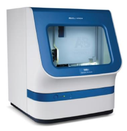


Tagging effective as demonstrated by transfection to (β)-actin, a gene involved in cell structure and integrity, and present in iPSC's.

- 5 days post-transfection with TrueTag™ homology directed repair knock-in at 10x magnification
- Flow cytometry confirmed 1.2% GFP

METHODS

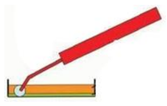
- iPSC's transfected with Neon electroporation using Invitrogen TrueTag™ Donor+cas9 RNPS (Day0)



- Junction PCR/sequencing to verify correct insertion of tag (when) (Day 15)

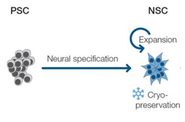
- Puromycin selection for 8 days

- Selected cells recovered in E8 media for 4 days

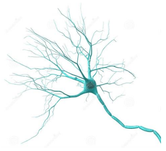


- Colonies manually picked and expanded for 20 days

- Junction PCR/sequencing repeated to verify clones

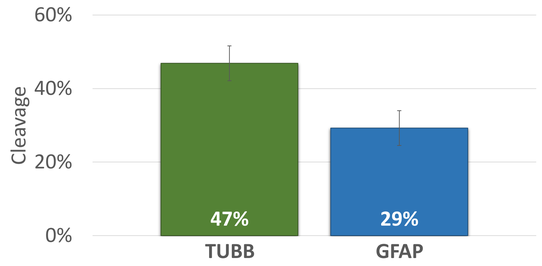


- Clones induced to neuronal stem cells (NSC) for 21 days
- Stained to verify neuronal markers and pluripotency



- Verified NSC's differentiated to astrocytes
- Verified GFP expression by imaging

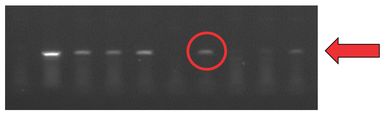
FIGURE 1: CRISPR cutting active as evidenced by GCD assay



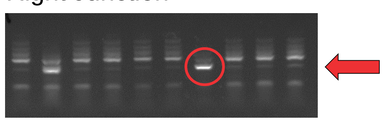
TrueCut Cas9 v2 with gRNA delivered by Neon electroporation generates cleavage at desired editing site

FIGURE 2: Junctions for GFAP Tag Verified by PCR & Sanger Sequencing

Left Junction



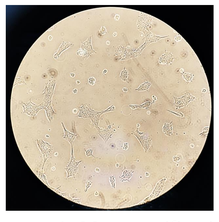
Right Junction



PCR indicated both junctions for GFAP clone (via puromycin enrichment) present. This was confirmed by sequencing in both left and right junctions.

FIGURE 3: Staining NSC's for Neuronal Markers and Pluripotency

Unstained NSC's



Stained NSC's

Passage 1, 20x magnification

GFAP-verified clone was induced to NSC state.

Discuss staining results

SUMMARY

As evidenced by sequencing, gene tagging was successful for both GFAP and TUBB. When the gene is turned on upon differentiation, the fluorescent marker will be visually scanned, indicating that the target gene is a useful marker of differentiation.

IMPLICATIONS

By fluorescently tagging genes in the iPS state then differentiating as desired, visualization will rapidly confirm cell lineage. You could potentially follow a more complex differentiation strategy by tagging multiple marker genes with various colors.

MATERIALS

All experiments described here were performed using

1. TrueTag™ Donor DNA GFP kit, TrueCut™ Cas9 v2 protein and TrueGuide™ synthetic guide RNA DNA for gene editing application
2. Neon™ Transfection System GeneArt™ for gene-editing efficiency
3. GeneArt™ Genomic Cleavage Detection Kit (GCD), and 3500 Series Genetic Analyzer™ for confirming editing efficiency and analysis
4. Attune NXT™ Flow Cytometry for protein knock-in efficiency, and BD FACSAria™ cell sorter for isolating iPSC's into clonal samples, using protocols described in the product manuals found on thermofisher.com.

ACKNOWLEDGEMENTS

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