APPLICATION NOTE

High throughput pooled CRISPR screening at single cell resolution

Introduction

The integration of single cell RNA sequencing with CRISPR-based gene editing technologies offers unprecedented opportunities for exploring complex biological pathways at an unparalleled resolution and scale. By enabling the simultaneous assessment of gene expression profiles and the functional impact of targeted CRISPR perturbations, this combined approach provides a powerful platform for investigating cellular processes, interactions, and heterogeneity within biological systems.

In this application note, we use the Scale Biosciences (Scale Bio) Single Cell CRISPR Guide Enrichment kit to interrogate the TNFα pathway in HEK293 cells using a pooled CRISPR knockout sgRNA library provided by Cellecta. This workflow facilitates the robust capture of CRISPR guides by utilizing a Cas9 scaffold-specific RT primer along with a CRISPR guide library enrichment step to increase the purity and sensitivity of the guide library and minimize sequencing costs. Our findings show that specific CRISPR guide capture along with highly sensitive transcriptomic data enables the identification of gene targets and their direct impact on the TNFα signaling cascade. Together, these results demonstrate that the Scale Bio Single Cell CRISPR Guide Enrichment kit can be used to profile CRISPR knockout experiments at scale.

Highlights

- Scale Bio's Single Cell CRISPR Guide Enrichment Kit and a pooled CRISPR knockout library from Cellecta enabled high-throughput screening of 192 sgRNAs targeting the TNFa signaling cascade.
- The workflow detected CRISPR guides with high sensitivity and purity, enabling lower sequencing burden and higher overall data quality.
- Simultaneous detection of CRISPR guide and gene expression profiles from ~135,000 cells with and without TNFa stimulation recovered key gene targets in a single experiment.
- Comprehensively characterize cellular pathways and responses with a large scale CRISPR screening approach.

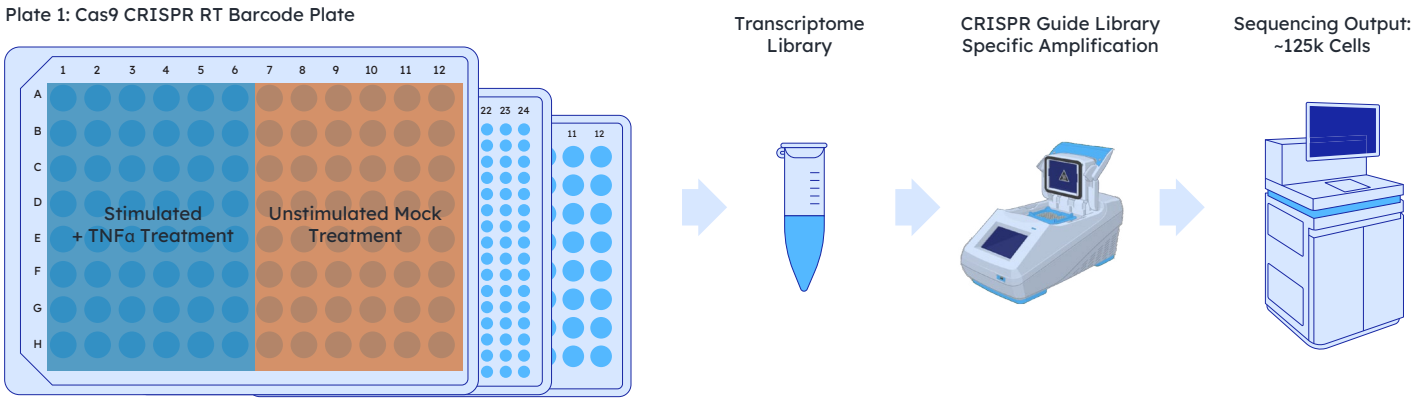


Figure 1. Experimental workflow. Scale Bio Single Cell RNA + CRISPR workflow depicting the even loading the two conditions onto the Cas9 CRISPR RT Barcode Plate. A single Final Distribution Plate was taken forward into RNA library generation targeting 125,000 cells for recovery. Diluted RNA library was used as input into the CRISPR guide library specific amplification PCR. RNA and CRISPR guide libraries were sequenced on a partial NovaSeq S4 run.

Materials and Methods

CRISPR Transduction

A pooled sgRNA CRISPR knockout library was constructed by cloning sgRNA-encoding oligos into a pRSGScribe lentiviral vector (Cellecta Inc, Mountain View CA) which is based on the CROP-seq design (Datlinger, et al., 2017). Functional sgRNA expressed from this vector enable targeted gene-specific CRISPR knockout as well as simultaneously being expressed as part of the polyadenylated mRNA transcript of the selection marker gene. This feature enables guide sequences to be captured along with other cellular mRNA using poly-A techniques (**Figure 2A**). HEK293 cells expressing the Cas9 enzyme were transduced with the pooled sgRNA library containing 192 guides targeting 48 genes in the TNFα pathway and a set of control guides. The cells were selected with puromycin and expanded for 10 days before being split into two samples. The samples were then stimulated with either TNFα or a mock (unstimulated) treatment for 72 hours, collected, and fixed using the Scale Bio Fixation kit (**Figure 2B**).

Single Cell Workflow

Fixed cells were divided evenly across the Cas9 CRISPR RT Barcode Plate of the Scale Bio CRISPR Guide Enrichment workflow, which utilizes an RT primer specific for the CRISPR guide scaffold*. Cells were loaded at a concentration of 20,000 cells per well and the single cell workflow was run following the Single Cell CRISPR Guide Enrichment Kit v1.1 protocol. A single Final Distribution Plate was taken for RNA library generation and cleanup, yielding an expected 125k cell output (**Figure 1**). Diluted RNA library was taken forward for CRISPR guide library specific enrichment utilizing hU6 as a priming sequence. RNA and CRISPR guide libraries were sequenced on a partial NovaSeq S4 flow cell and analyzed with the Scale Bio Seq Suite pipeline.

*For more information on compatible vectors and primer sequences see Vector Compatibility with CRISPR Kit (Document 1097102).

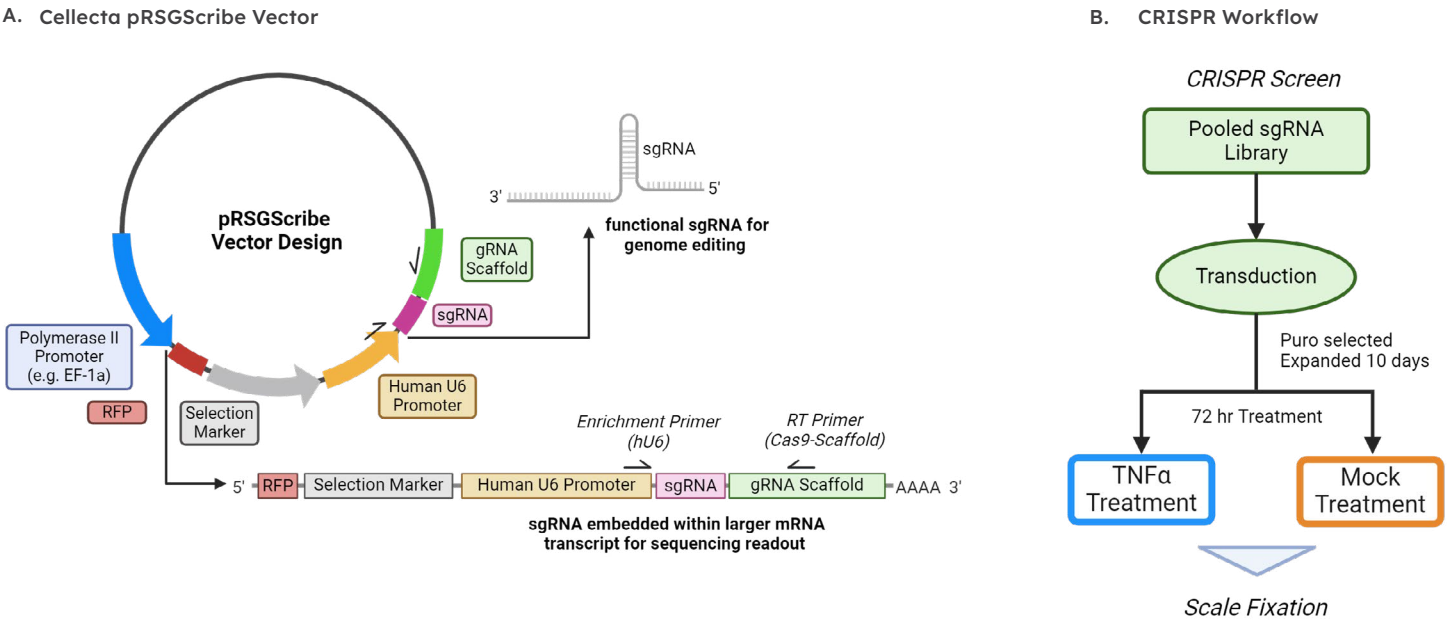


Figure 2. CRISPR Perturbation design. **A)** pRSGScribe lentiviral vector design. **B)** Diagram depicting the CRISPR screen workflow

Results

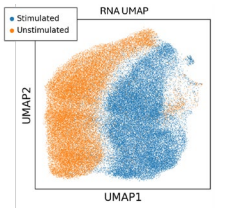
RNA library sensitivity and cell recovery

The RNA transcriptomic and CRISPR Guide libraries were sequenced and processed using the Scale Bio Seq Suite pipeline. To assess the quality of the transcriptomic data, we examined the cell recovery and RNA sensitivity across the two treatment conditions. At extremely shallow sequencing (5% saturation) we observed expected cell recovery of 134,000 cells across the final sample split evenly between TNFα treated (71,000) and unstimulated (63,000) conditions (**Figure 3A**). Furthermore, we observed strong transcript and gene recovery in both conditions (**Figure 3A**). UMAP clustering revealed distinct clusters based on differential gene expression for TNFα treated and unstimulated samples (**Figure 3 B and C**).

A.

RNA Library Metrics	TNFα Stimulated	Unstimulated
Cells called	71,551	63,060
Reads Mapped to Genome	91.60%	92.50%
Reads Mapped to Transcriptome	85.60%	85.70%
Reads in cells	87.80%	87.40%
Median unique transcript at 10000 total reads per cell	6,457	6,329
Median Genes per Cell	4,684	4,063
Sequencing Saturation	5%	5%

B.



C.

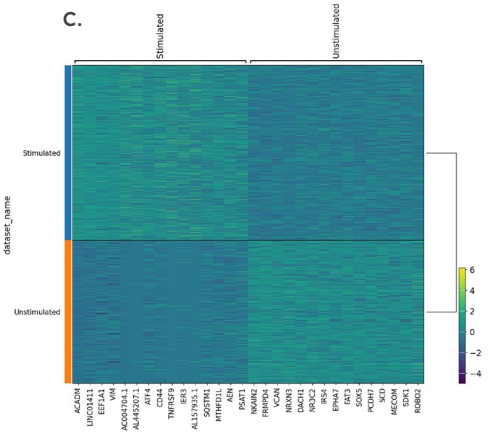


Figure 3. RNA library sensitivity and cell calling. A) Metrics for the RNA library separated by treatment conditions. **B)** UMAP plot showing distinct clusters overlayed by treatment condition. **C)** Cell by gene heatmap of the differentially expressed separated by treatment condition.

CRISPR Guide Library Sensitivity

To assess the effectiveness of the CRISPR perturbation, we evaluated the sensitivity of the CRISPR guide library. Our analysis showed a high percentage of reads with correct CRISPR barcodes (89%), allowing for a high percentage of cells assigned to a guide, with 77% in the TNFα stimulated condition and 70% in the unstimulated control (**Figure 4A**). Each of the 192 guides was assigned to at least 10 cells across both treatment conditions, demonstrating consistent guide representation between conditions (**Figure 4B**). To further evaluate the uniformity of the CRISPR perturbation we counted the number of cells assigned to each of the 48 gene targets within the 192-guide library (**Figure 4C/D**). We found a comparable number of perturbed cells for each guide target in both the TNFα stimulated condition and the unstimulated condition. These results indicate that a single Scale Bio CRISPR Guide Enrichment workflow provides sufficient cell numbers and sensitivity to effectively profile large CRISPR screens.

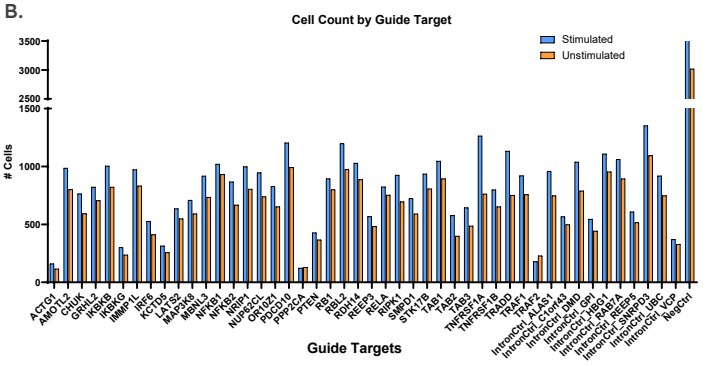
TNFα Pathway analysis

Using the transcriptomic and CRISPR guide library data, we examined the biological effects of the gene disruptions. By comparing the transcriptomic profiles of the CRISPR edited cells under TNFα stimulated and unstimulated conditions, we were able to identify potential modulators and downstream effectors of the pathway. We first identified the general gene expression signature of TNFα stimulation by determining the most differentially expressed genes between the stimulated and unstimulated treatments in control cells (cells that received a negative control guide). We then plotted the expression of these genes across all perturbations in both treatment conditions (**Figure 5A**), which identified several gene targets that appeared to diminish TNFα response in the TNFα treated condition upon knockdown.

A.

CRISPR Guide Library Metrics	TNFα Stimulated	Unstimulated
Total Reads	396,390,042	283,167,783
Incorrect Guide Reads	11%	11%
Correct Guide Reads	89%	89%
Mean reads/cell	5,012	4,059
Mean UMIs/cell	53	33
%Passing Cells with a Guide	77%	70%

B.



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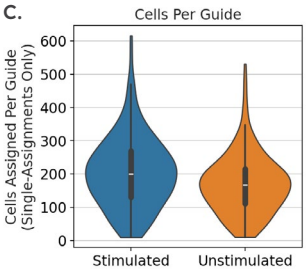

D.

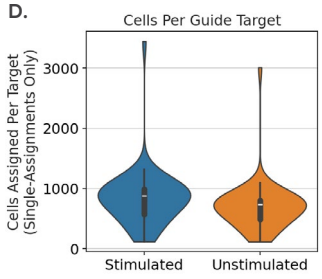

Figure 4. CRISPR guide library sensitivity and guide assignment. A) Metrics for the CRISPR Guide Library separated by treatment condition. **B)** Bar graph plotting the number of cells for both treatment conditions by guide targets (48 targets). **C)** Violin graph showing the assigned cells to individual CRISPR guides (192 guides) by treatment condition. **D)** Violin graph showing the assigned cells to guide targets (48 targets) by treatment condition.

To further analyze these guide targets within our large single cell dataset, we averaged the gene expression of the cells of each treatment condition into pseudobulk profiles (**Figure 5B**). Unsupervised clustering of these pseudobulk profiles revealed differences within the TNFα stimulated group (**Figure 5B**). Notably, seven of the guide targets with the most significant impact to the stimulation-response signature clustered separately from the remainder of the TNFα stimulated group. Overlaying the inferred TNFα pathway activity we observed that the cluster containing the 7 guide targets has reduced TNFα pathway activity, consistent with their involvement in the TNFα signaling cascade (**Figure 5C**). Although these perturbations affected the TNFα treated group's response, they remained clearly distinct from the unstimulated group, suggesting the presence of parallel TNFα signaling pathways or indirect effects of stimulation. Overall, our study profiled 135,000 cells using a 192-guide library and identified a subset of genes that can be further explored for their roles in pathway modulation in response to TNFα stimulation.

Conclusion

In this study, we have demonstrated the effectiveness of a combined Single Cell RNA and CRISPR approach to investigate the TNFα signaling pathway. By leveraging the Scale Bio Single Cell CRISPR Guide Enrichment kit along with the Cellecta's designed CRISPR library, we achieved robust guide capture and assessment of specific genes in the pathway. Overall, we profiled 135,000 cells with a 192-guide library, demonstrating the scalability and robustness of this approach for large scale CRISPR knockout experiments.

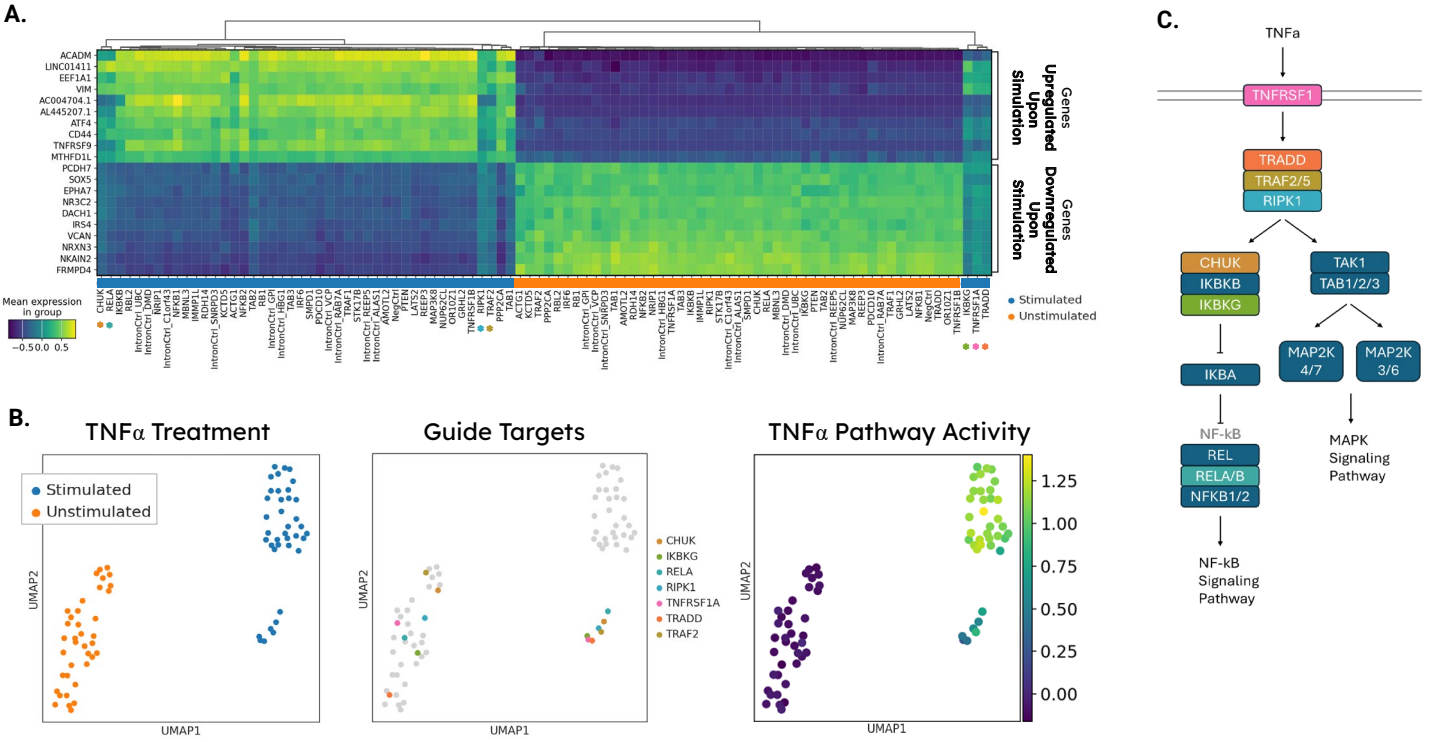

Figure 5. TNFα pathway analysis. A) Heatmap of normalized gene expression for the genes that are most differentially expressed between stimulated and unstimulated control cells (signatures of TNFα response). Expression values are the average within each perturbation-treatment. Dendrogram at top shows hierarchical clustering of conditions using all gene. **B)** Left: UMAP of pseudobulk profiles from each perturbation-treatment combination derived by averaging gene expression across the cells of each group. Center: Guide targets/perturbations that appear to impact response to stimulation. Right: Pathway activity scores for each pseudobulk profile based on an independent model of pathway-responsive genes (PROGENy). **C)** Simplified schematic of TNFα pathway, with impactful perturbations highlighted.

To learn more about ScaleBio's CRISPR Guide Enrichment Kit, visit https://scale.bio/single-cell-crispr-guide-enrichment-kit/