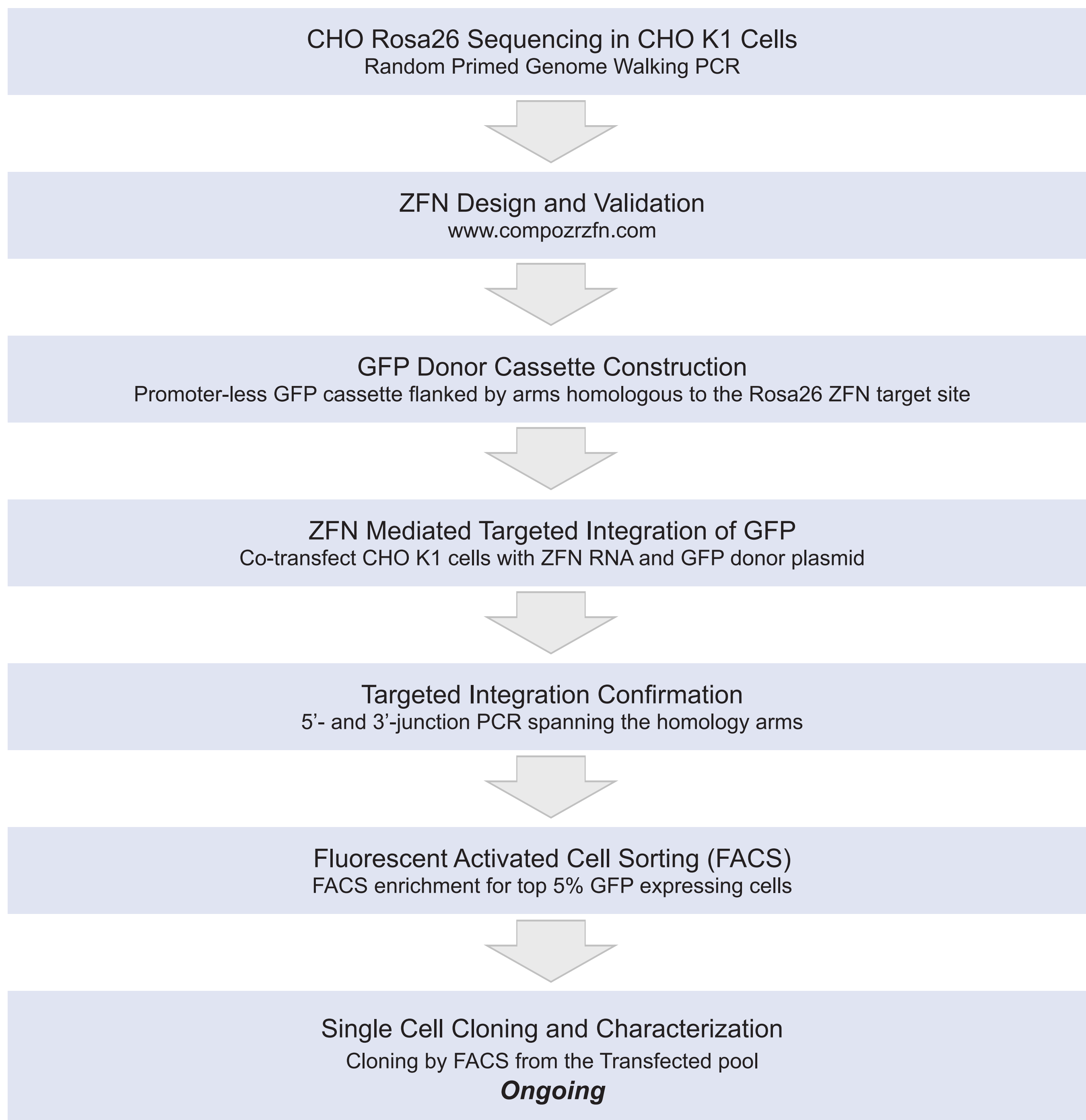


Purpose

The biopharmaceutical industry has expressed considerable interest in targeted integration in CHO cells for therapeutic r-protein production applications. In previous studies we have used ZFN's for site specific integration of short exogenous DNA fragments (recombinase sites, restriction enzyme sites) into the CHO genome. In this study we characterize the Rosa26 locus, a well known genomic locus used for targeted integration in rodent systems. We also examine targeted integration of a larger exogenous DNA fragment containing a green fluorescent protein (GFP) expression construct into the ROSA26 locus.

CHO Rosa26 ZFN Mediated Targeted Integration Workflow



Results

CHO Rosa26 Sequence Homology with Mouse

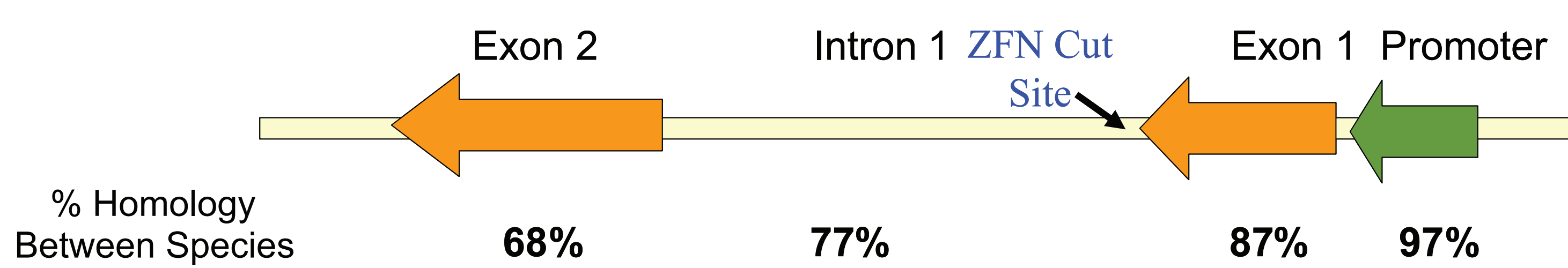


Figure 1. CHO Rosa26 Identification and ZFN Design

CHO ROSA26 locus was identified using BLASTN homology search and random gene priming methods. We characterized an 8 kb DNA sequence that corresponds to the homologous mouse Rosa 26 genomic region. Overall homology of the 8 kb genomic region flanking the Rosa 26 loci was 68% compared to mouse but specific regions such as the Rosa26 promoter (97%) had much higher sequence similarity. CHO Rosa26 ZFN pairs were designed against intron1 (similar to the target region of previously validated mouse ZFN's).

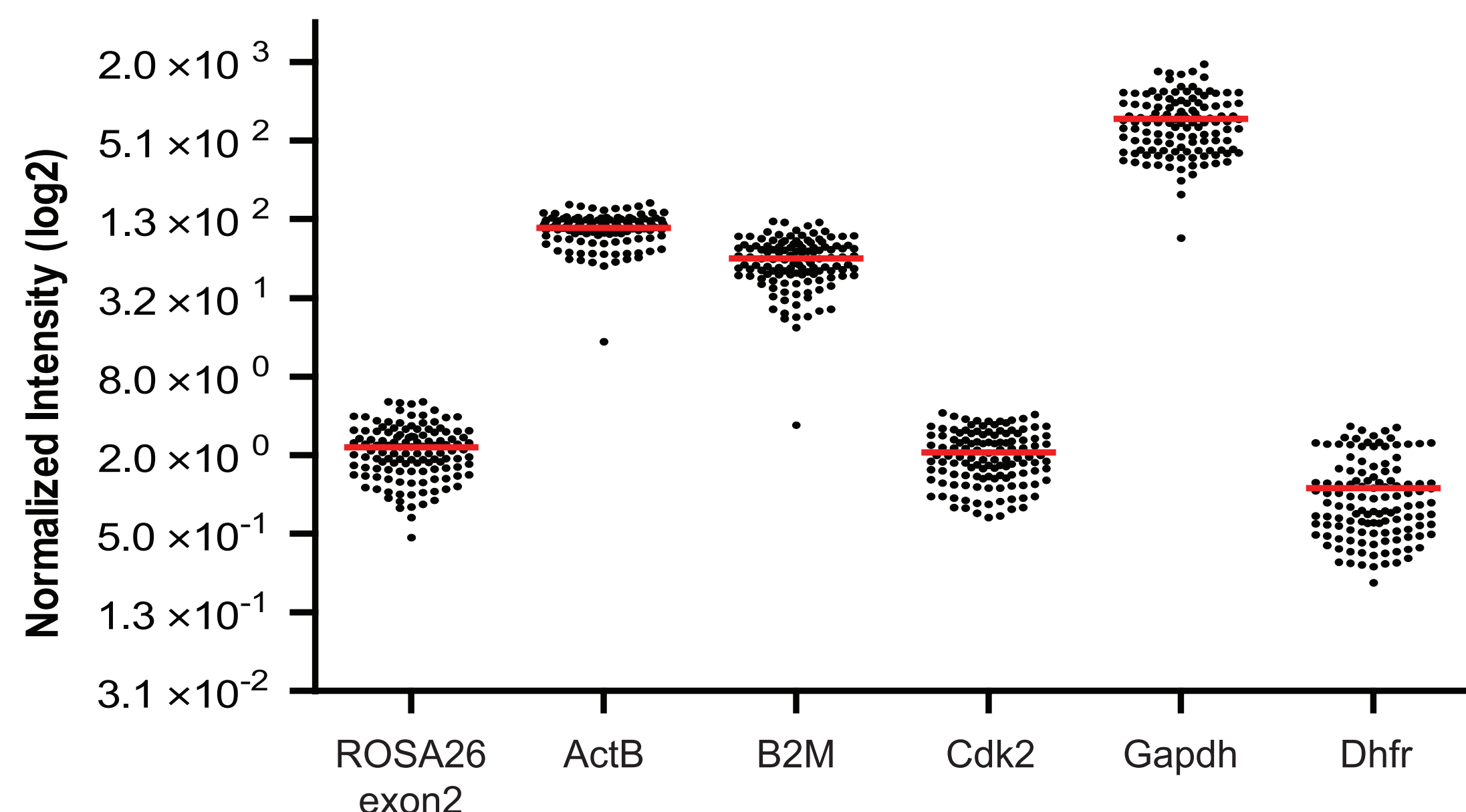


Figure 2. CHO Rosa26 Relative Expression

Relative expression of the putative Rosa26 exon 2 in microarray studies representing 24 unique gene expression studies from a variety of CHO cell culture phenotypes. The graph depicts normalized raw fluorescent intensity values of probes of Rosa26 and several well characterized housekeeping genes.

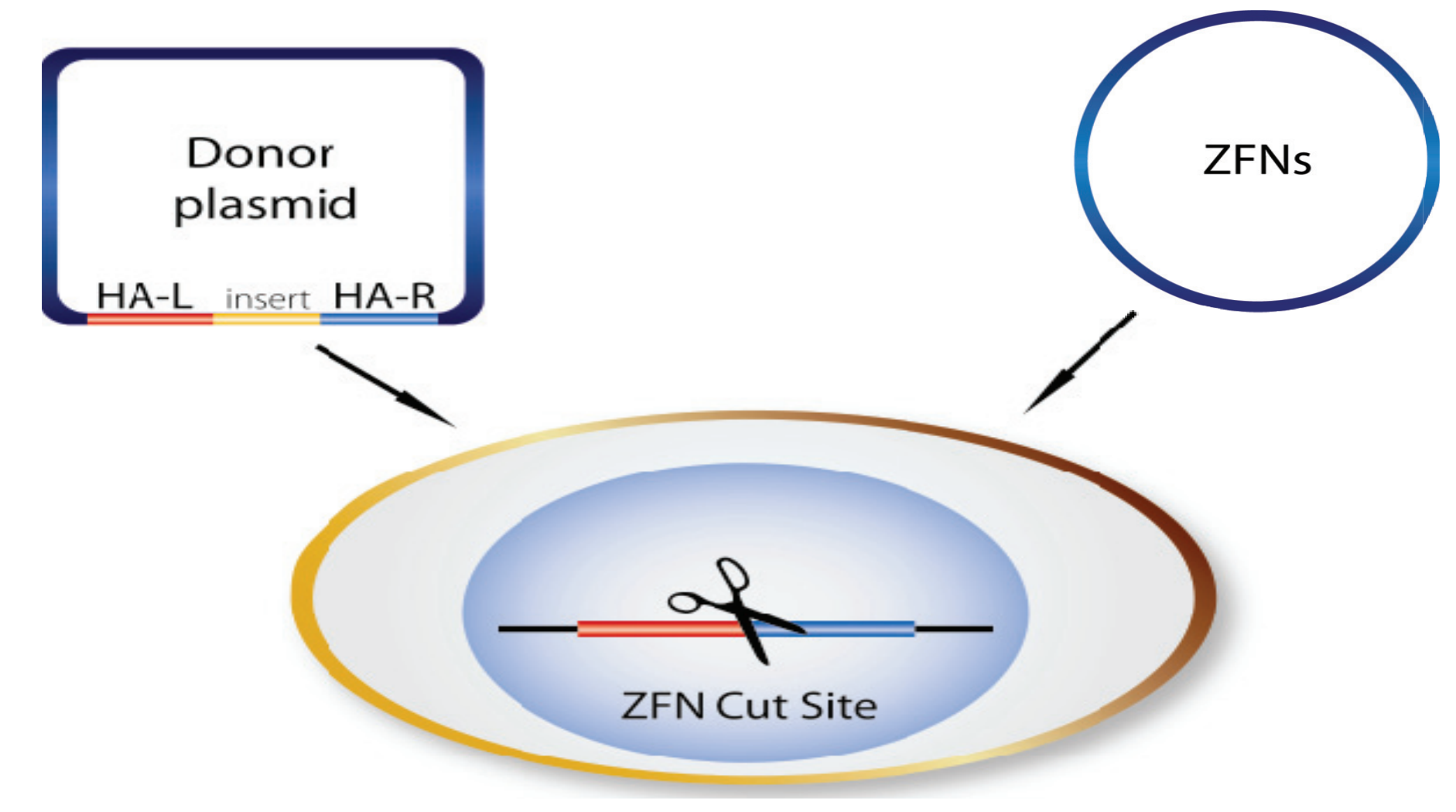


Figure 3. Schematic of ZFN Mediated Targeted integration

The ZFN's and Donor plasmid are co-transfected into the host cell line. The activity of the ZFN pair creates a double stranded break in the specific genomic sequence. Through homologous recombination the exogenous donor DNA construct (see Figure 4) is integrated into the host genome.

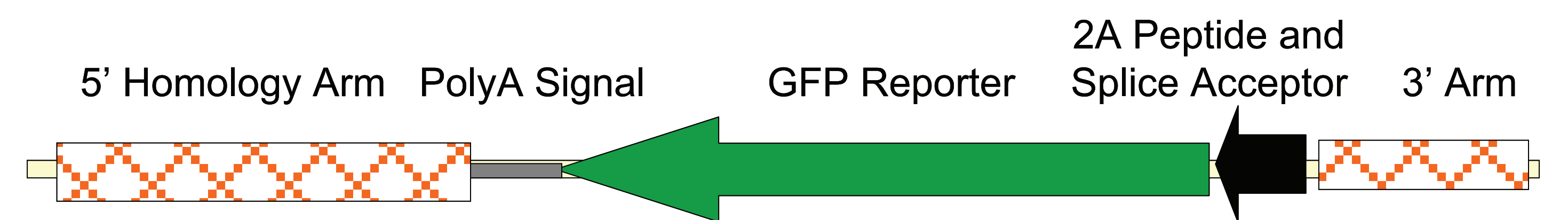


Figure 4. GFP Donor Construct with Rosa26 Homologous Arms

GFP expression cassette lacking a promoter and flanked by arms homologous to the Rosa26 ZFN target site. The GFP Open Reading Frame (ORF) is preceded by a splice acceptor and 2A peptide to enable GFP expression driven by the endogenous Rosa26 promoter.

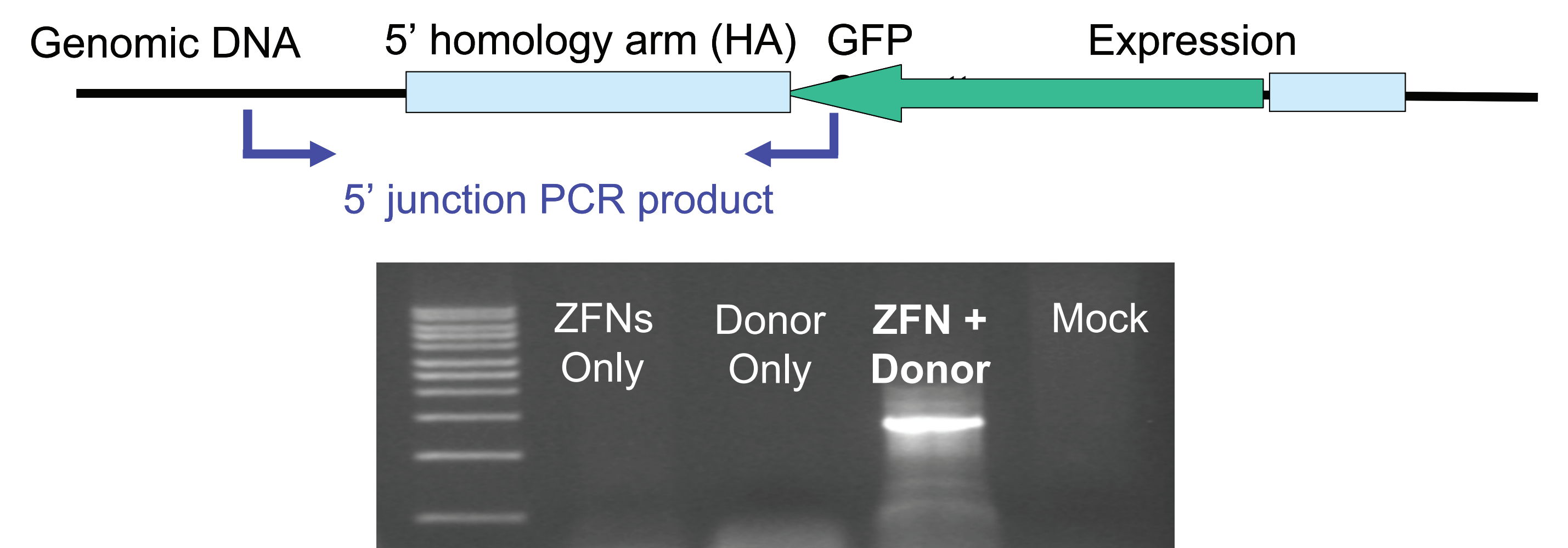


Figure 5. Targeted Integration Validation in the Transfected Pool

Ten day post-transfection genomic DNA was purified and a PCR amplified using primers that span the 5' junction of the GFP construct and the CHO genomic region adjacent to the integration site. DNA sequencing of the subsequent PCR product confirmed the integration event (PCR fragment contained sequence from 3' region of the GFP expression cassette adjacent to the targeted CHO Rosa26 genomic sequence)

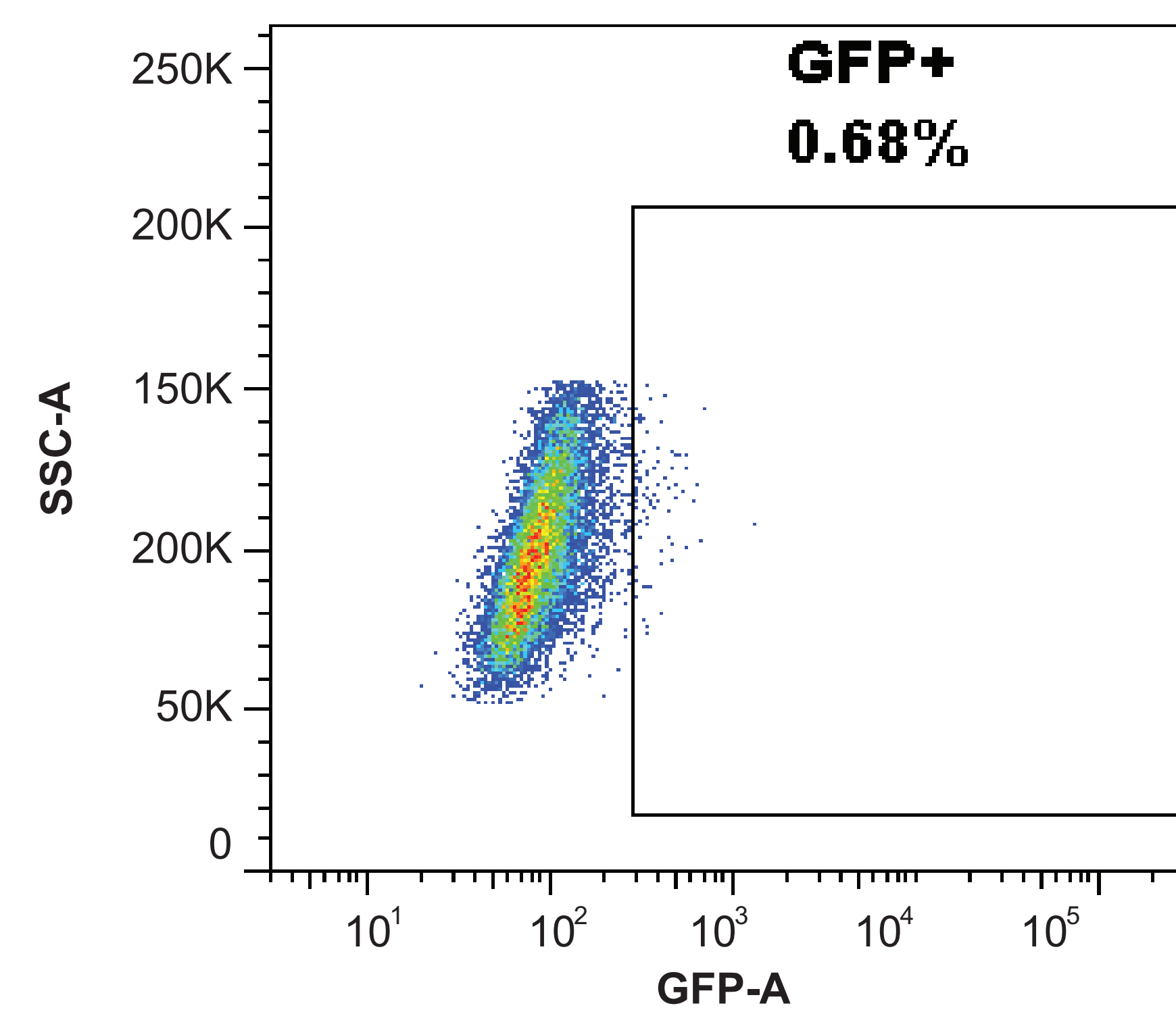


Figure 6. FACS Analysis of GFP expression

Following the positive junction PCR results the pool was tested for GFP expression. The transfected pool was sorted based on the top 0.5% of GFP expression (x-axis), collected and expanded. This pool of cells will be further characterized for targeted integration events.

Conclusion and Discussion

- We have identified and characterized the Rosa26 locus in CHO K1 cells. CHO Rosa26 is partially homologous to the mouse Rosa26 coding sequence. Rosa26 promoter had 97% homology with the mouse locus, whereas exon and intron sequences show decreased homology
- ZFNs were designed and validated targeting the putative intron 1 of the CHO Rosa26 locus. Targeted integration at the CHO Rosa26 locus was indicated in the transfected pool based on the junction PCR and sequencing results
- The transfected pool was further enriched using FACS for GFP positive cells. A large single cell cloning activity is underway to further characterize integration events. Previous experimental results indicate that the integration frequency (targeted vs. random) is dependant on the genomic loci targeted, ZFN cutting activity and donor size and format
- Ongoing studies continue to examine additional transcriptionally active and stable CHO genomic loci. We are developing methodology to integrate larger r-protein constructs, such as heavy and light chain monoclonal antibodies. As we continue to characterize integration events we will adjust conditions to optimize targeted integration in CHO cells

Acknowledgements

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