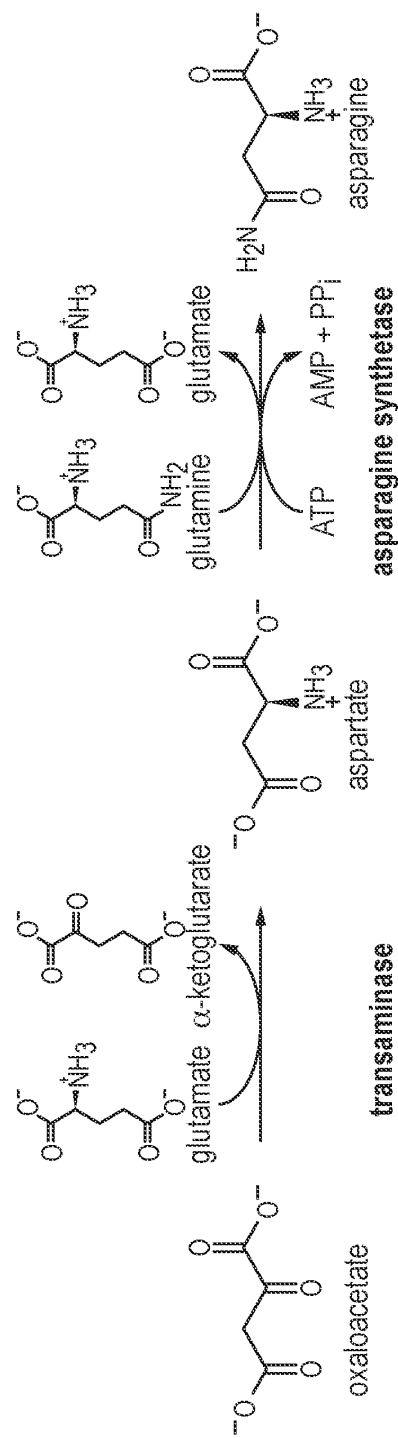




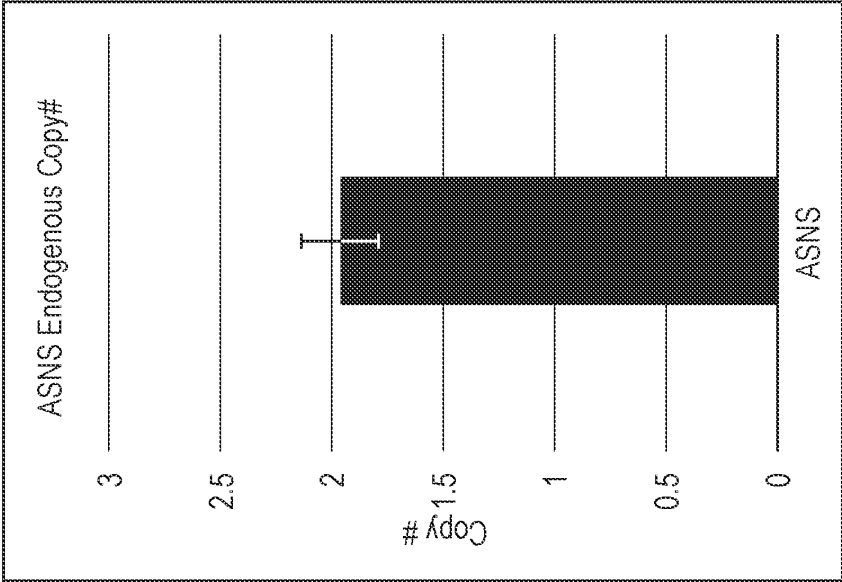
The final step of asparagine biosynthesis is completed via a single pathway that requires the non-redundant enzyme asparagine synthetase (ASNS). In the absence of functional ASNS protein the cells require an external source of the amino acid asparagine.

Figure 1

ATGTGTGGCAATTTGGGCCCTCTTTGGCAGCGATGACTGCCTTCCGTGCACTGCTGAGTGCAATGAAGATTGCACACAGAGGG
 CCAGATGCATTTCTGTTTGAAGATGTCAATGGATAACCAACTGCTGCTTGGATTTCACCGTTGGCCGTGGTTGACCCCTGTT
 TGGATGCAGCCAATAAGAGTGAAGAAATACCTTATCTGTGGCTCTGTACAATGGTGAATTTACAACCAAGGCGCTACAA
 CAACGCTTTGAATTCGAGTACAGACCAATGTGATGGTGAGATTACTCTGATGACAAAGGAGGTATCGAGCAAACCA
 TTTGATGTTGGATGGGTGTTGCATTTCATCTGCTGGACACTGCCAACAAAGAAAGTTTCTGGGCAGAGATACCTATGGAGT
 CAGGCCCTTGTAAAGCCATGACAGAAAGATGGATTCTGGCTGTGTTTCAGAAAGCTAAAGGGCTGTTTCTTAAAGCACTC
 CACAACTCCCTTCTTAAAGTGGAGCCCTTCTCTGGACACTACGAAGTTTGGATTAAACCAAAATGGCAAAGTTGCAICT
 GTGAGATGTTGAATACCATCACTGCAGAGATGAACCACTGCACGCCCTTACGACAGCGTGGAGAACTCTTCCAAGGCTTT
 GAGTTAGAAACGGTGAAGAGCAATCTTCGTATCTGTTTGACAGTGTCTCAGGAAGCGCTTGATGACGGACAGGAGGATCGG
 CTGCCCTTTATCAGGGGCTGGAATCGAGCTTGGTTGCCGCCCTCCCTGCTGAAGCAACTCAAGGAGGCTCAAGTACAGTATCC
 ACTCCAGACATTTGCAATTGGCATGGAAGACAGCCCTGATCTACTAGCCGCTAGAAAGGTGGCAAATATATTGGAAGCGAGCA
 TCACGAAGTCTTATTTAATTCTGAAGAAGGCATTCAGGCCCTGGATGAGTCAATTTTCTTGGAAACTTACGACATTACAACAG
 TTCGAGCACTGTAGGTATCTAATTTCCAAGTATATTCGGAAGAACACAGACAGCGTGGTGATCTTCTCTGGAGAAGGTC
 AGATGAACCTTACACAGGGCTACATATAATTTCCACAAGGCTCCTTCTCCAGAGAAAGCAGAGAGGAGTGAAGACTGCTGA
 AGAACTCTACCTGTTTGAATGTTCTCCGAGCCGACCGAACTACTGCTGCCACGGTCTTGAACTGAGAGTCCCGTTTCTGGATCA
 TCGGTTTTCCTCTTACTTGTCACTGCCACCAGAAATGAGAAATCCAAAATGGATAGAAAACATCTCCTTCGAGAGACG
 TTTGAGGACTCCAACTGTACCCAAAGAGATTCTCTGGAGACCAAAAGAGCCTTCAGTGAATGGGATCACCTCAGTTAAGAAC
TCTGGTTTAAAGATTCTACAGGATTATGTTGAACATCAGTTGATGATGAATGATGCAACAGCAGCCAGAAAGTTTCCCTTCA
 ATACTCCAAAACTAAAGAGGCTATTACTACCGCCAGATCTTTGAACGCCATTACCCAGCGCGGTGATTGGCTGACCCATTAT
 TGGATGCCCAAAGTGGATCAATGCCACCGATCCTTCTGCCGAACCTCTACCCACTATAAGTCAAGTCAAGCTGCAAAAGCTTAG

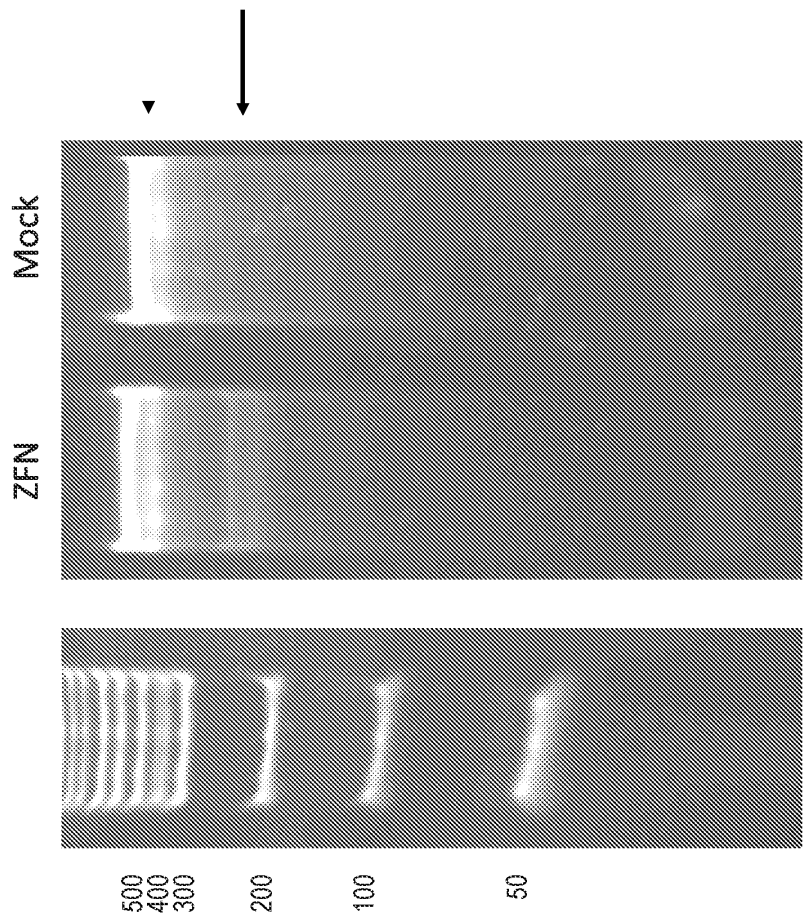
cDNA sequence of ASNS in CHOZN^a GS^{-/-}.

Figure 2



Copy number analysis via ddPCR of the endogenous Asparagine Synthetase gene (ASNS).

Figure 3



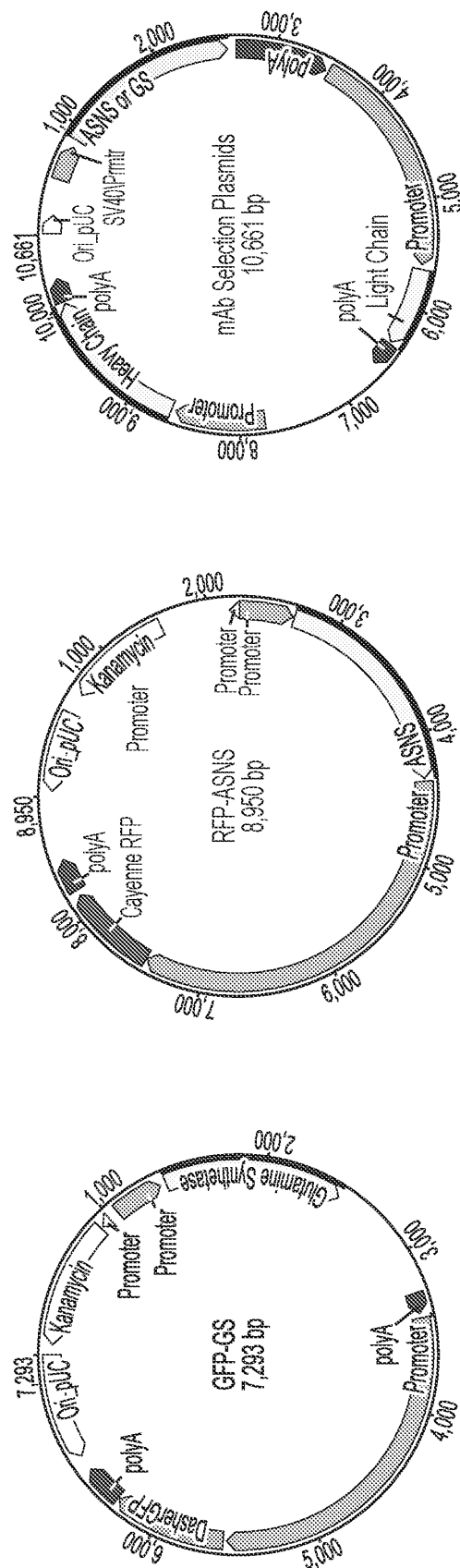
ZFN cutting activity was evaluated with supplementation of Asn into the media. The arrowhead indicates the expected size of an ASNS PCR amplicon while the arrow specifies two bands resulting from a Surveyor mismatch repair assay indicating that a genetic modification occurred at the ZFN cutting site.

Figure 4

Clone 26 F3 Allele 2: GCCTTCAGTGAIGGGATCACCTCAGTTAAGA CTCCTGGTTTAAGATTCTACAGGATT

Genetic changes in the coding sequence of ASNS were detected via amplicon sequencing of two independent clones and result in frameshift mutations and inactivation of ASNS protein.

5010

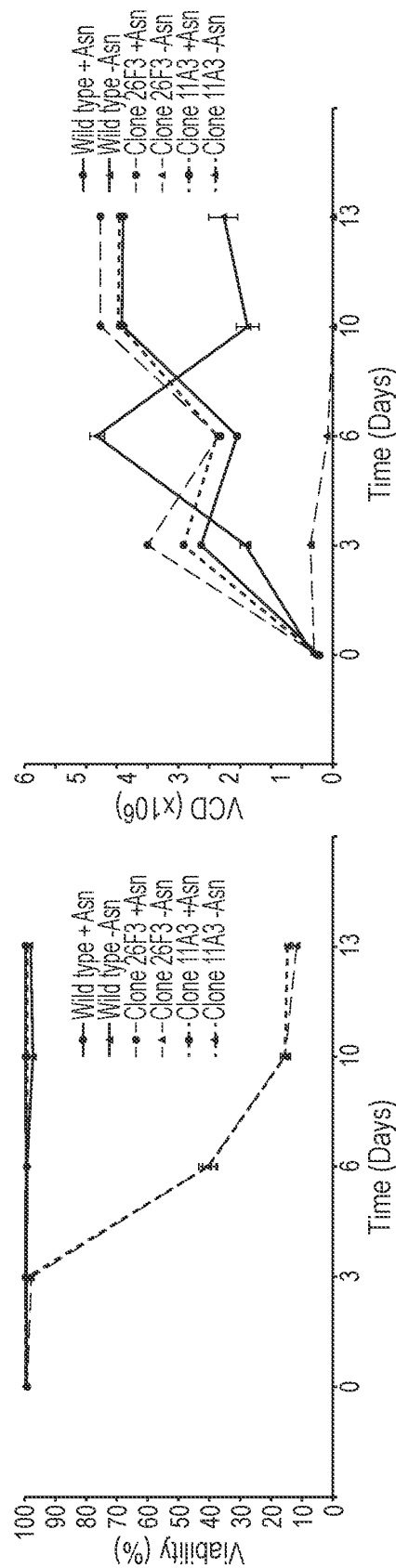


Vectors were designed to allow for selection of -GFP positive cells using a glutamine-based selection system, -RFP positive cells using an asparagine-based selection system as well as development of secreted recombinant proteins via development of two similar vectors that contain a mAb heavy chain, light chain and either the ASNS or GS coding sequence.

Figure 6

SUBSTITUTE SHEET (RULE 26)

Figure 7
ASNS Endogenous Expression Control Sequence (Promoter)



ASNS KO clones 11 A3 and 26 F3 are unable to survive and grow without supplementation of Asn in the media.

Figure 8

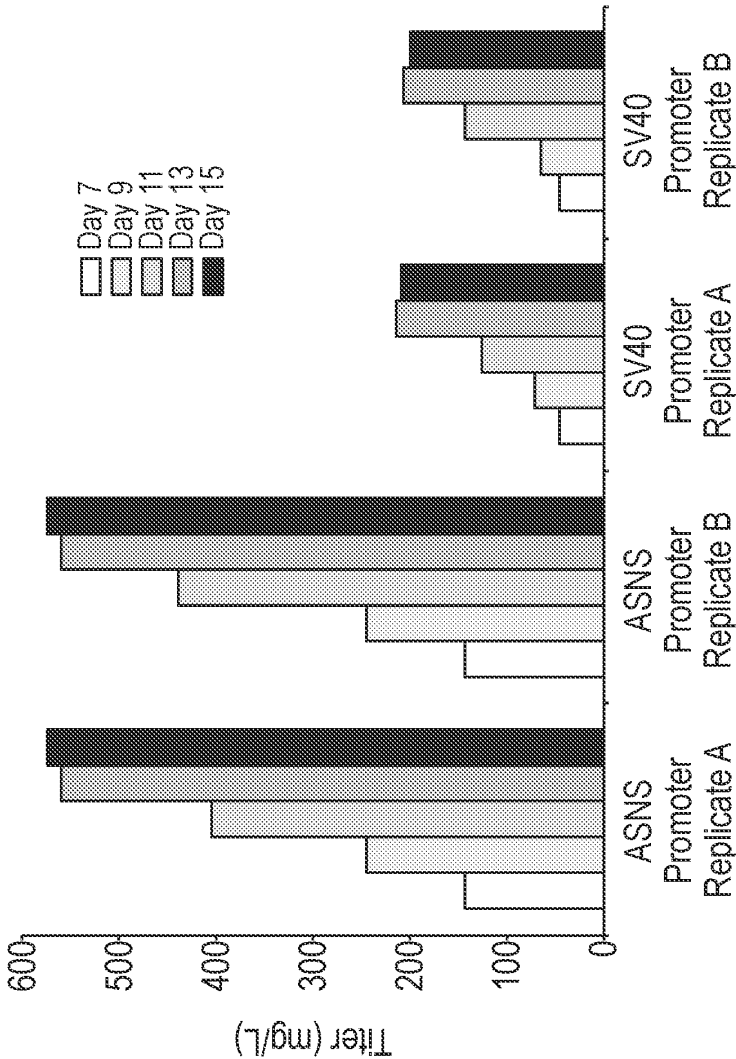
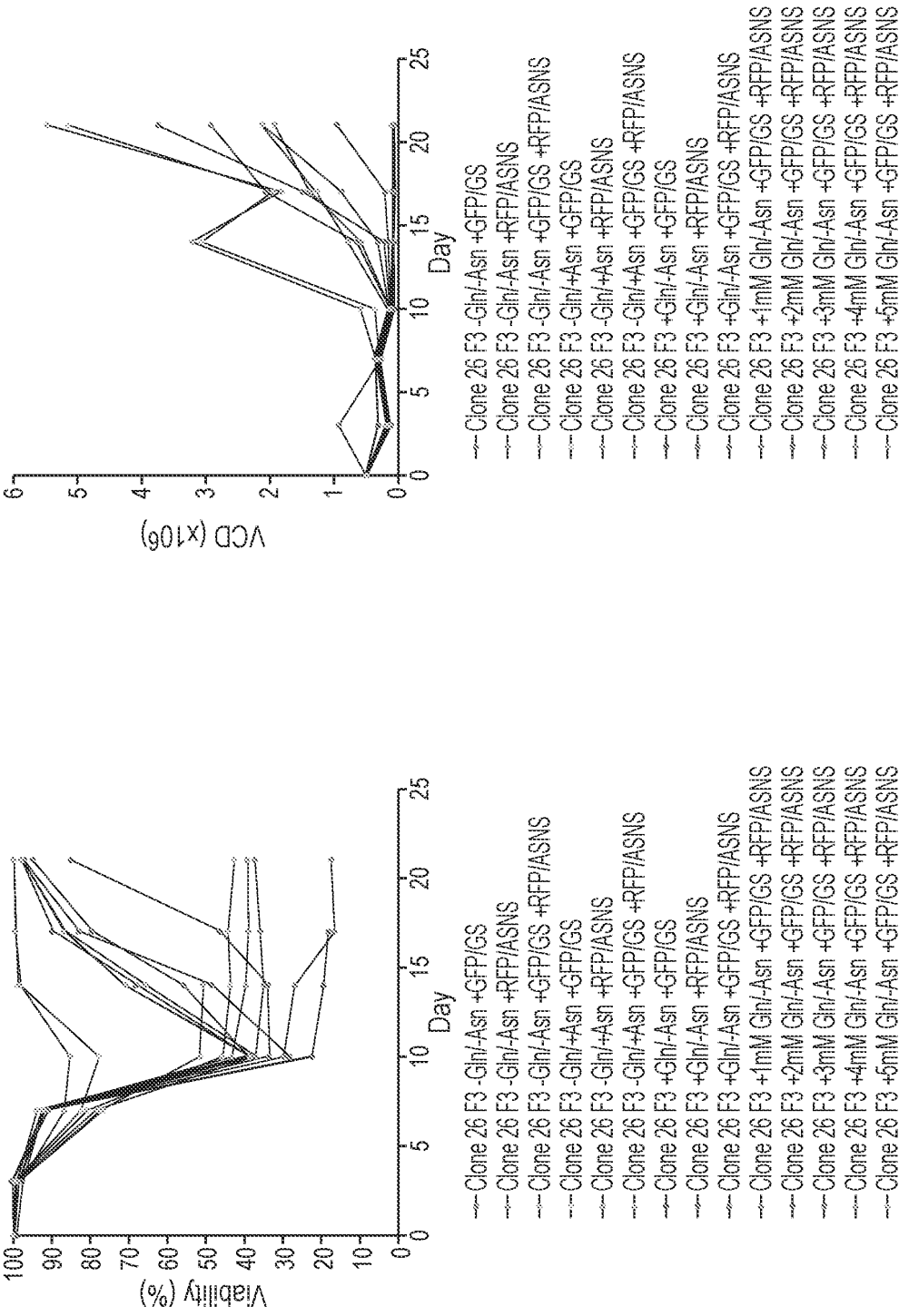


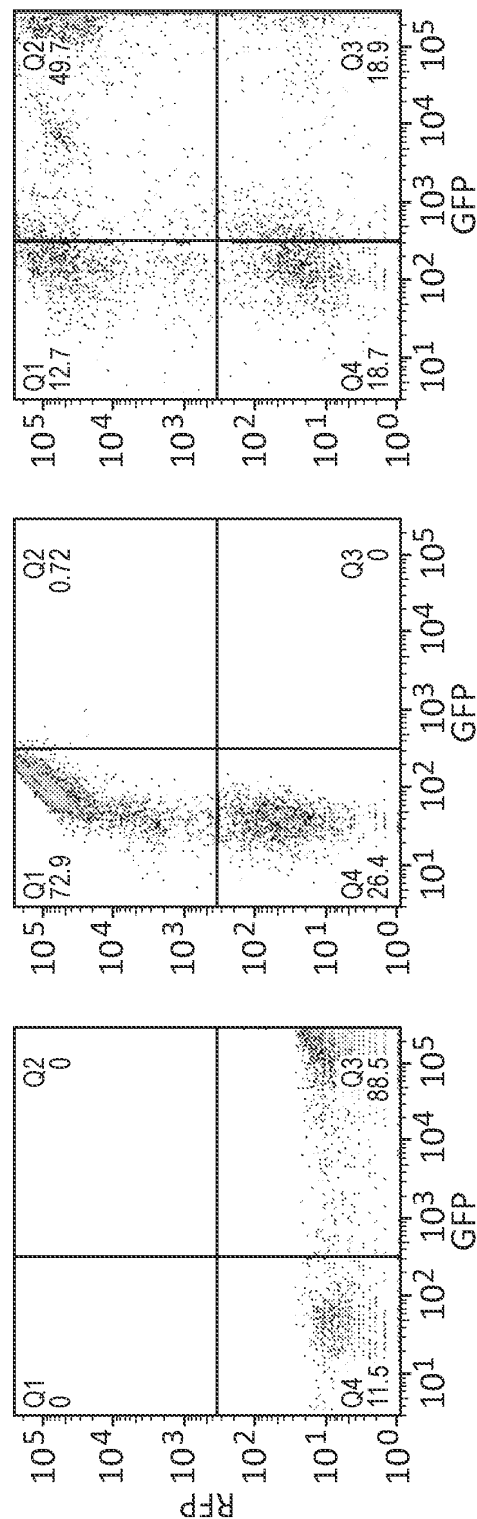
Figure 9

CHO cells with genetically disrupted GS and ASNS genes transfected with an IgG+ASNS driven by either an endogenous expression control sequence (ASNS Promoter) or an SV40 promoter and grown in asparagine deficient media supplemented with 6mM glutamine. IgG expression levels were found to be ~3x higher when ASNS was driven by an ASNS promoter as opposed to an SV40 promoter.



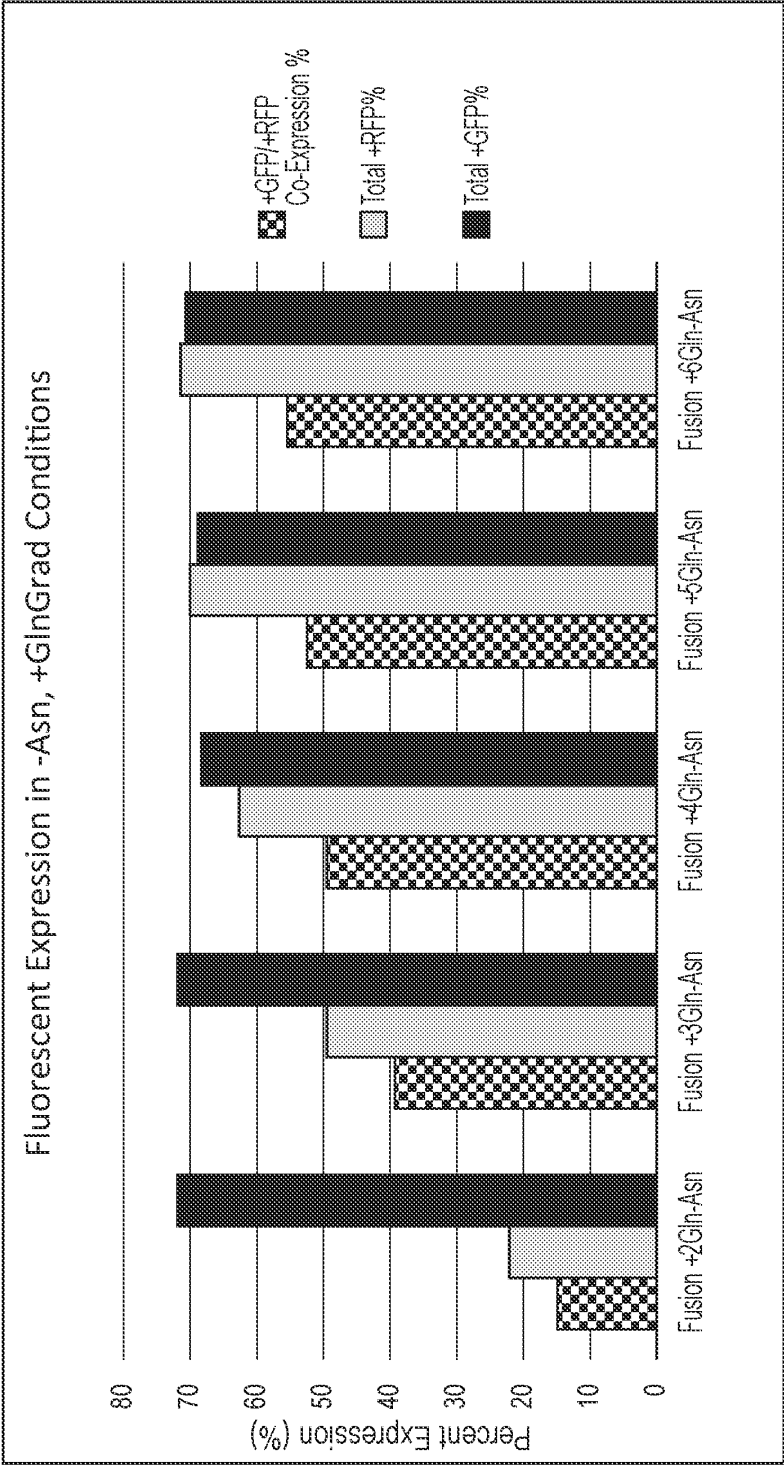
Growth and viability of pools expressing GFP, RFP or GFP + RFP using GS, ASNS or GS + ASNS selection, respectively.

Figure 10



CHO cells with genetically disrupted GS and ASNS genes transfected with a plasmid containing GFP+GS expression cassettes were selected in glutamine deficient media and express GFP, but do not express RFP (far left). CHO cells with genetically disrupted GS and ASNS genes transfected with a plasmid containing RFP+ASNS expression cassettes were selected in asparagine deficient media and express RFP, but do not express GFP (middle). CHO cells with genetically disrupted GS and ASNS genes co-transfected with GFP+GS and RFP+ASNS and grown in asparagine deficient media supplemented with low levels of glutamine express high levels of both GFP and RFP.

Figure 11



CHO cells with genetically disrupted GS and ASNS genes co-transfected with GFP+GS and RFP+ASNS and grown in asparagine deficient media supplemented with a gradient of glutamine show no loss of GFP expression (black). Total RFP expression (grey) increases precipitously with an increased concentration of Glutamine supplementation, as does GFP/RFP co-expression (checkered).

Figure 12

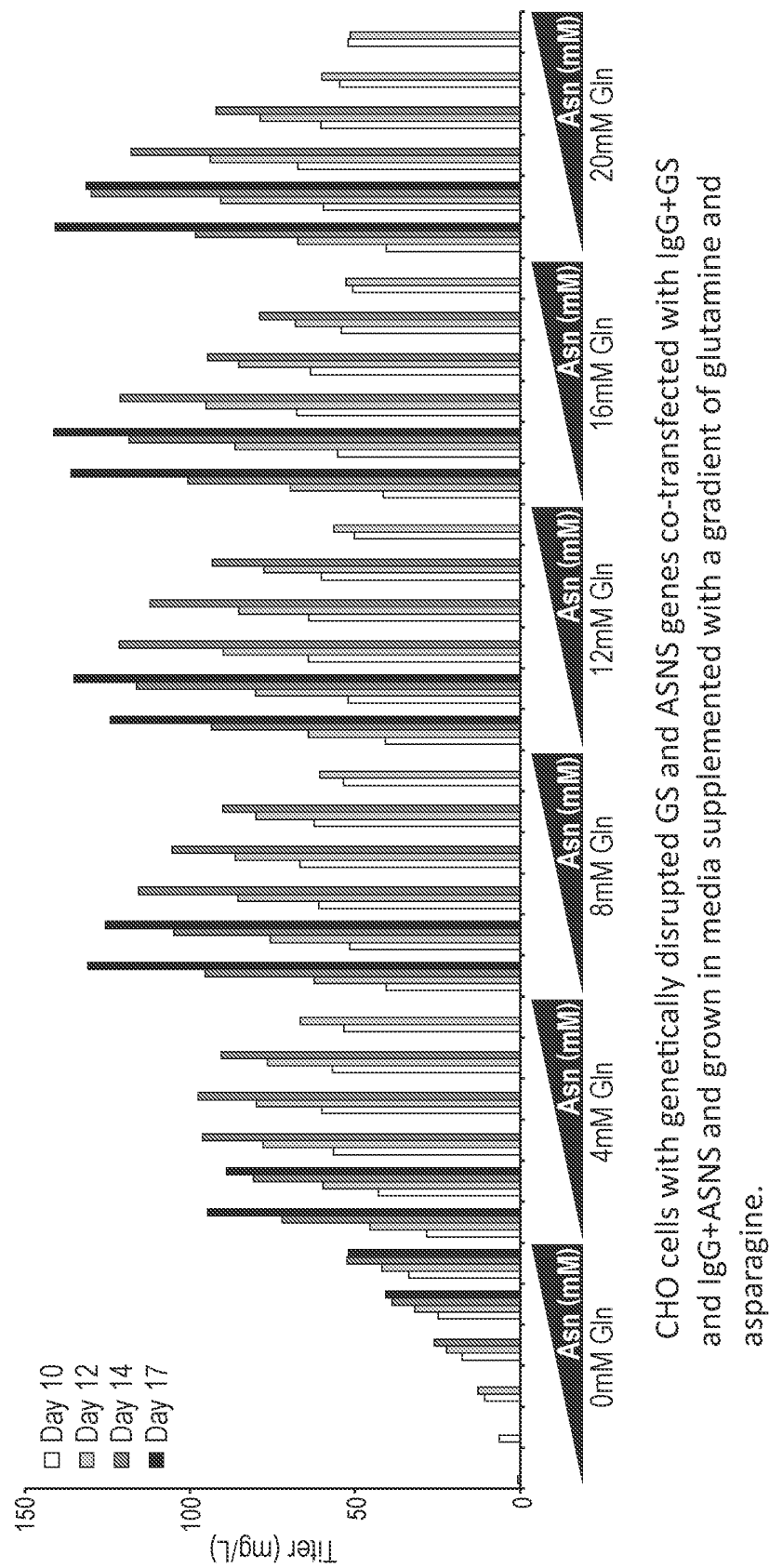


Figure 13