Design and Development of Liver Targeted Synthetic Promoters for Gene Therapies.

Introduction

The liver is a crucial target organ for therapeutic transgene expression in several gene therapies of inherited diseases such as Hemophilia A and B, Phenylketonuria, Fabry disease, ornithine transcarbamylase (OTC) disease, familial hypercholesterolemia (FH), and acquired diseases such as liver cancers and hepatitis [1]. This derives from its central role in the regulation of metabolism, protein synthesis, and the systemic presence of vital proteins [2]. Achieving optimal transgene expression for desired therapeutic outcomes in liver gene therapies requires promoters that can drive transcriptional outputs at safe and efficacious doses of the transgene vector. Additionally, in many liver gene therapies, there is the need to de-target expression from critical tissues where therapeutic transgene expression might be otherwise deleterious.

Generally, vectors such as recombinant adeno-associated viral vectors (rAAVs) offer serotype-based tissue-tropic delivery of transgene payloads into specific tissue types [3]. However, there is a need for transcriptional targeting to further restrict transgene expression to specific tissue/cell types to achieve a sufficient level of specificity for safety. Viral promoters such as hCMV (human cytomegalovirus IE-1 promoter), CAG (CMV enhancer and the chicken beta actin promoter), SV40 (Simian Virus 40 promoter), and native mammalian promoters such as EF1-a (Elongation factor 1-alpha promoter) exhibit ubiquitous activity across diverse cell/tissue types, unsuitable for applications where tissue specific expression is paramount [3,4]. Additionally, gene therapy developers have a limited repertoire of promoter strengths available to optimise transgene expression levels for different gene therapy requirements.

Several liver targeted hybrid promoters (conjoining large fragments of different context-specific natural regulatory elements) have been applied in liver gene therapies already. Unlike conventional approaches that rely on natural or hybrid promoters, synthetic promoters offer a distinct advantage because they can be tailored to therapeutic requirements. This flexibility is essential since liver gene therapies address a wide spectrum of genetic and acquired diseases, each with unique transgene expression needs. A ‘one-size-fits-all’ strategy cannot meet these diverse requirements. Expanding the repertoire of promoter designs enables customization for disease-specific expression levels, vector packaging constraints, and immunogenicity considerations, thereby improving both performance and applicability.

Objectives

To address the need for highly specific liver expression, SynGenSys developed liver targeted synthetic promoters built *de novo* from units of *cis*-active sequence elements which include transcription factor response elements (TFREs) or *cis*-regulatory modules (CRMs) conforming to predefined functional and structural specifications with the potential of better performance than existing hybrid and naturally occurring promoter sequences for liver targeted expression.

The aim was to develop liver targeted synthetic promoters in two broad categories:

i. Liver specific synthetic promoters exhibiting high activity, relative to the hCMV promoter, in liver hepatocytes and minimal activities in skeletal muscle (<5% of the activity of the hCMV promoter) at >20-fold ratio of activity between liver and skeletal muscle. In addition, we aimed for the liver specific promoters to also exhibit lower activities relative to the hCMV promoter benchmark in other off-target tissues such as the kidney and cerebral cortex.
ii. Liver active synthetic promoters (liver max) exhibiting a range of activity in liver hepatocytes, reaching and exceeding the

activity of the hCMV promoter benchmark in liver hepatocytes.

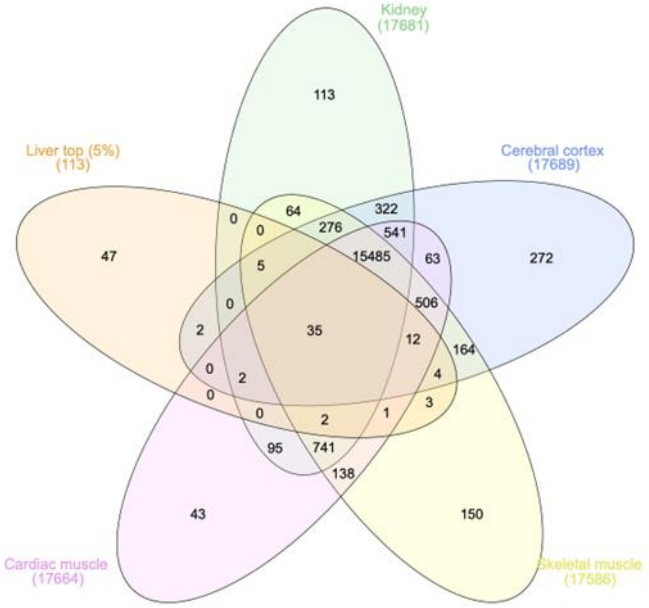

Figure 1: *Identification of human liver specific genes expression elements relative to key off target tissues.*

Specifications for structural properties such as sequence length, CpG content, and CpG islands were targeted toward achieving promoters with shorter lengths, higher activity-to-size ratios, and reduced CpG content compared to the hCMV promoter benchmark.

Design of Liver specific synthetic promoters.

Liver specific TFREs were identified through computational analysis of the transcriptional landscape of human liver to allow identification of *cis*-regulatory elements driving high level expression in liver, and a similar analysis of specific off-target tissues **(Figure 1)**. Computational analysis was supplemented with review of pre-existing in-house experimentally validated expression data for selected TFREs to achieve a comprehensive view of the elements responsible for liver specific gene expression, their relative relationships and interactions. A proprietary, rules-based approach was applied to the assembly of a liver specific promoter library, with candidate TFREs selected to provide sequence diversity and high specificity in the liver, whilst accounting for spatial and positional relationships between individual TFREs and the expressed gene. A first library was designed with set TFRE selection rules and the aim to test and validate performance of TFRE selection, position, and combination relative to the target goals of specificity and expression strength. As such, first library promoter design is not directly intended to achieve maximal performance, but to inform a learning feedback process and provide data to support functional design for a second, enhanced library to be designed.

First library - assay of selected liver specific synthetic promoters.

Assay of the first library of liver specific synthetic promoters identified sequences exhibiting up to ~0.5-fold the activity of the hCMV promoter, and minimal activities in C2C12 and HEK293 cell lines **(Figure 2)**.

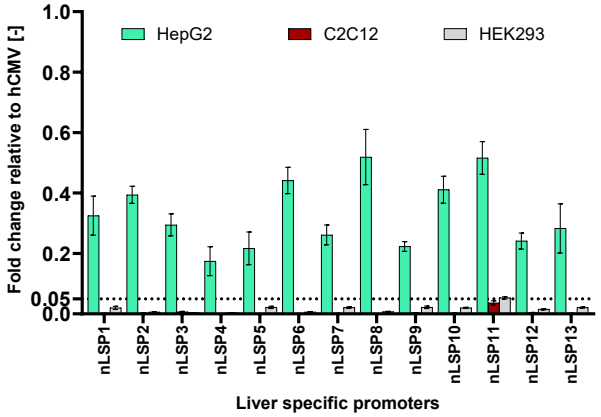

Figure 2: *Assay of activity of first library of liver specific synthetic promoters in liver cell line HepG2 (green bar), skeletal muscle cell line C2C12 (red bar) and HEK293 cell line (blue bar). Activities of promoters in each cell line are normalised relative to the activity of the hCMV promoter in each cell line. Promoters exhibit between 17% and 55% of the hCMV promoter activity in the target HepG2 liver cells. Dotted line shows that all promoters display less than 5% of hCMV promoter activity in the off-target C2C12 muscle cells and HEK293.*

Design of second library of Liver active synthetic promoters.

Data from the assessment of library one was interrogated to determine TFRE selection, combination and positional information to inform the design of a second promoter library. To create a second library of 30 liver active synthetic promoters, the heterogeneity of the promoter sequence composition was enhanced through combination of TFREs exhibiting high activity in the

liver HepG2 cell line, measured with in-house reporter assays, and supplementary pan-active TFREs.

Second Library - assay of selected liver active synthetic promoters.

Promoters displaying activities up to ~2-fold the hCMV promoter in the HepG2 liver cell line were identified following assay of the second library of liver-active synthetic promoters **(Figure 3)**.

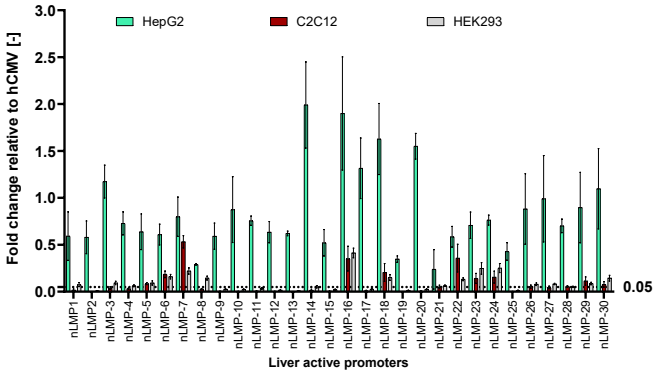

Figure 3: *Assay of liver active synthetic promoters in liver cell line HepG2 (green bar), skeletal muscle cell line C2C12 (red bar) and HEK293 cell line (blue bar). Activities of promoters in each cell line are normalised to the activity of the hCMV promoter. Maximal expression has been increased to ~2- fold above that of the hCMV promoter activity in the target HepG2 liver cells whilst activity below 5% of hCMV promoter in the off-target muscle C2C12 cells and the kidney HEK293 cells has been maintained in a substantial portion of the promoter designs.*

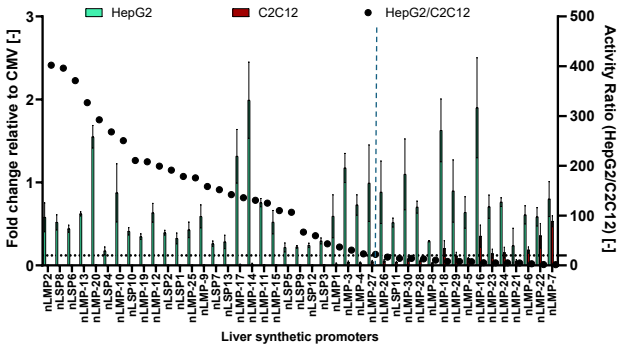

Figure 4: *A ranking of all 43 liver targeted synthetic promoters (black dot) in decreasing order of HepG2/C2C12 activity ratio showing 28 promoters with activity ratio > 20 and 15 promoters with activity ratio < 20.*

All 43 synthetic liver promoters were ranked based on a ratio of HepG2 to C2C12 activity (*i.e.* HepG2/C2C12 activity ratio), to identity the synthetic promoters displaying specificity in the liver according to the predefined selection criteria **(Figure 4)**.

Out of the 43 promoters tested, 28 met the predefined criteria of exhibiting a greater than 20-fold activity ratio between the liver cell line HepG2 and the skeletal muscle cell line C2C12, while maintaining less than 5% of the hCMV promoter activity in C2C12. Furthermore, all these promoters showed less than 10% activity in HEK293 cells, a feature that can be advantageous for viral vector production, as undesired therapeutic transgene expression in manufacturing cell lines may negatively impact yield **(Figure 5)**.

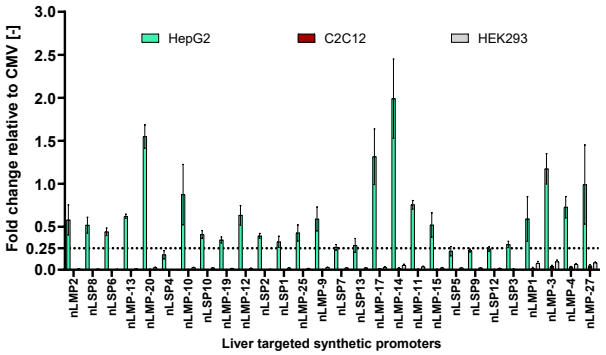

Figure 5: *Selection of 28 liver targeted synthetic promoters which exhibit over 20-fold ratio of activity between the target liver HepG2 cell lines and off-target skeletal muscle C2C12 cell lines, and less than 5% and 10% the activity of the hCMV promoter in C2C12 and HEK293 cell lines, respectively.*

Assay of selected liver active synthetic promoters in primary human hepatocytes.

Seven of the 28 liver targeted synthetic promoters were further assayed in human primary hepatocytes (HUCPG) to allow comparison with expression strength characteristics observed in HepG2 cell lines. A high degree of correlation was observed between both models ($r = 0.9853$, $p < 0.0001$, two tailed), with an eight-fold range of activity **(Figure 6)**.

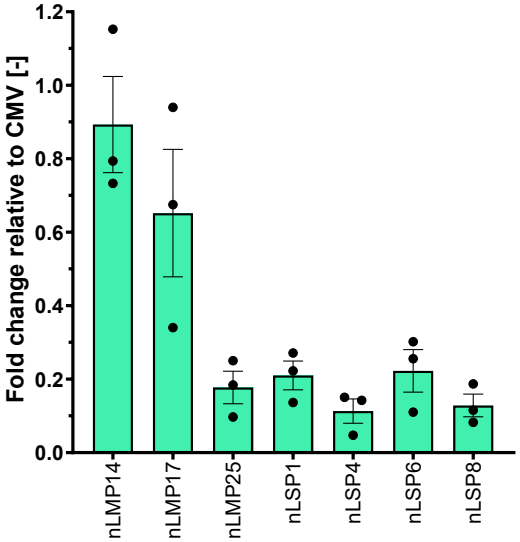

Figure 6: *Assay of the transcriptional activity of 7-liver targeted synthetic promoters – nLMP14, nLMP17, nLMP25, nLSP1, nLSP4, nLSP6, nLSP8 in a primary human hepatocyte cell model (HUCPG). Promoters exhibit an ~8-fold range of activity relative to CMV.*

In vivo validation of liver targeted synthetic promoters.

The liver targeted synthetic promoters were next assayed for *in vivo* activity using AAV9-based gene delivery in BALB/c mice. All AAV cloning, production, and purification, animal preparation, and AAV administration, whole body *in vivo* bioluminescence assays, tissue collection, RNA and DNA extraction, and RT-qPCR and qPCR were conducted by a specialized contract research organization. The relative influence of core and enhancer promoter elements was assessed through combination of suitable core promoters with liver targeted synthetic enhancers nLSP4 and nLMP14. These synthetic enhancers were chosen to represent the broadest range of *in vitro* liver activity in both the HepG2 cell line and HUCPG primary cells **(Figure 3, 4, 5 & 6)**.

The hCMV enhancer was also assessed with these core elements to provide a benchmark and control for both liver specificity and expression levels driven by synthetic enhancer sequences, and to determine whether the core elements alone improve liver-specific targeting and expression. *In vivo* bioluminescence imaging and quantification of the luciferase mRNA transcripts at 3-weeks post AAV9 injection revealed that the two core promoters in combination with the CMV enhancer do not exhibit specificity for liver expression relative to the hCMV core with the CMV enhancer **(Figure 7A,B)**. Despite the lack of overall specificity up to 2.3-fold and 2.7-fold increase in liver expression was achieved with liver core 1 (LC1) and liver core 2 (LC2), respectively relative to with the CMV core **(Figure 7A,B)**.

Luciferase based measurement of the nLMP14 and nLSP4 synthetic enhancers with LC1 and LC2 promoters, showed significantly improved specificity in liver expression (negligible expression in the skeletal and cardiac muscles) compared to constructs with the CMV enhancer **(Figure 77C)**. Additionally, higher liver expression was observed with the nLMP14 synthetic enhancer than the nLSP4 synthetic enhancer with both core promoters thus recapitulating the pattern of expression observed in *in vitro* assays with both HepG2 cell lines and HUCPG liver hepatocyte primary cells **(Figure 7D)** and confirming that our synthetic liver targeted enhancers actuate liver specific expression. For both nLSP4 and nLMP14 synthetic enhancers, LC1 yielded higher liver targeted expression than lLC2, as similarly observed with the CMV enhancer **(Figure 7D)**.

Next, the use of an intronic sequence in the 5'UTR was explored to further improve the level of liver targeted expression from our synthetic enhancers with the stronger LC1. For this purpose, a short, viral intron sequence was used. Up to ~1.6-fold increase in expression in the liver was achieved with the nLSP4-LC1+intron construct relative the nLSP4-LC1 construct without the small intron **(Figure 6E,F)**. Up to ~3.2-fold increased expression in the liver was achieved with nLMP14-LC1+intron relative to the nLMP14-LC1 construct without the intron **(Figure 7E,F)**.

Overall, the synthetic promoters combined with the intron achieved ~3-fold and ~6-fold higher expression in the liver compared to the CMV promoter (with the CMV core). These constructs also displayed markedly improved specificity of liver expression over the CMV enhancer with all three cores **(Figure 7E,F)**, demonstrating the critical influence of enhancer elements in defining both specificity and expression strength, and showcasing the ability to tune expression strength

while maintaining specificity through modification of the enhancer element.

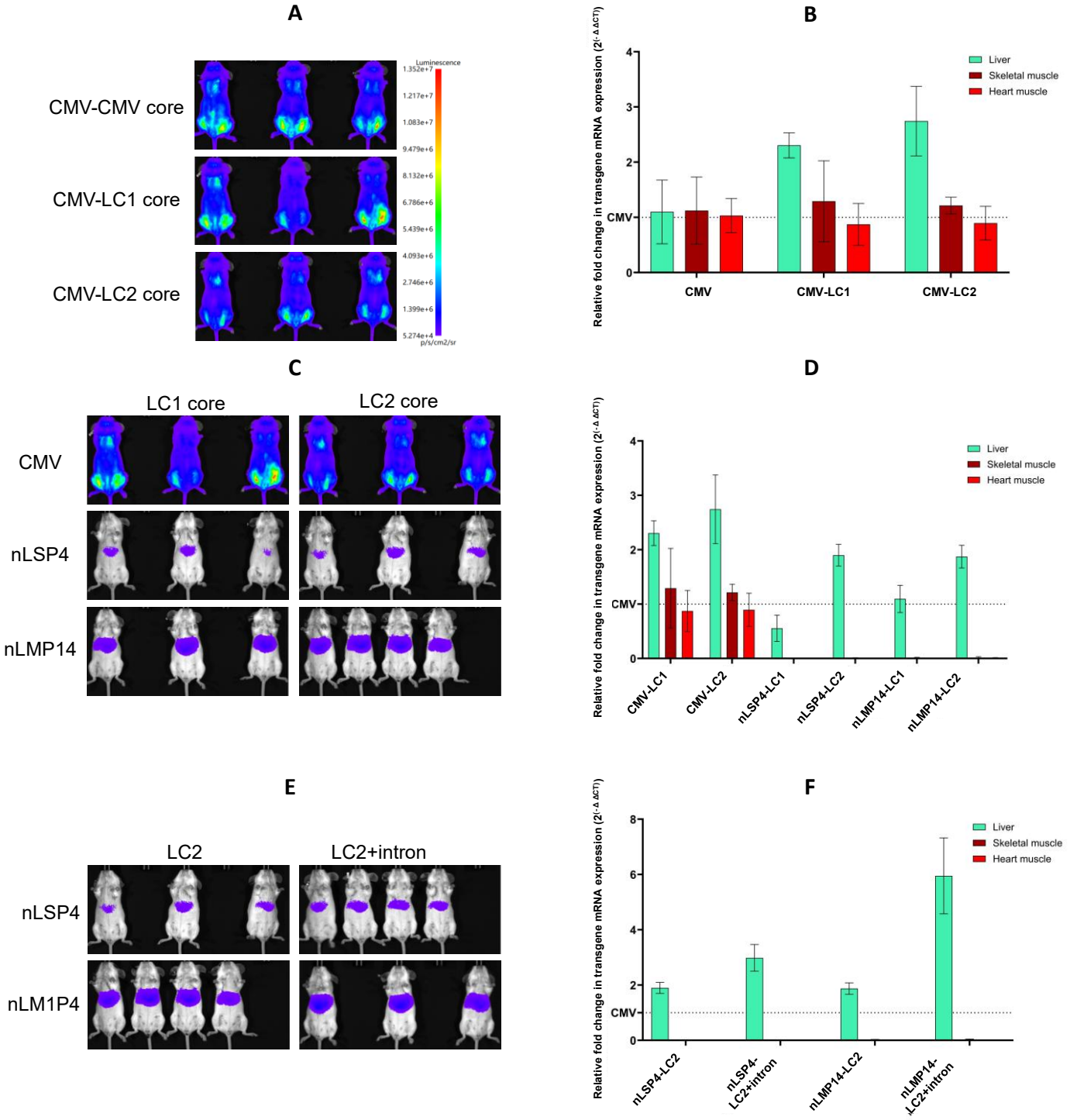

Figure7: *In vivo bioluminescence and mRNA quantification of expression of [A,B] CMV enhancer with 3 different core (CMV core, LC1), [C,E] CMV enhancer, nLSP4 and nMLMP14 synthetic enhancers with 2 different cores (LC1 , and LC2), [E,F] nLSP4 and nMLMP14 synthetic enhancers with just LC2 core and with LC2 and a short intron.*

Conclusion and Future perspectives

We have demonstrated the capability to develop liver targeted synthetic promoters conforming to predefined functional requirements of transcriptional activity and specificity in liver tissue against pre-defined off tissues including skeletal muscle and kidney / virus manufacturing cell line HEK293, with functional validation using *in vitro* cell line models and *in vivo* AAV delivered transduction, through a process of computationally guided mining, identification, characterisation and selection of component *cis*-active response elements **(Figure 8)**.

This capability provides the basis for the development of tissue specific promoter constructs, engineered for user-defined requirements of expression strength, on and off target activity and suitability for deployment with AAV based gene therapy approaches.

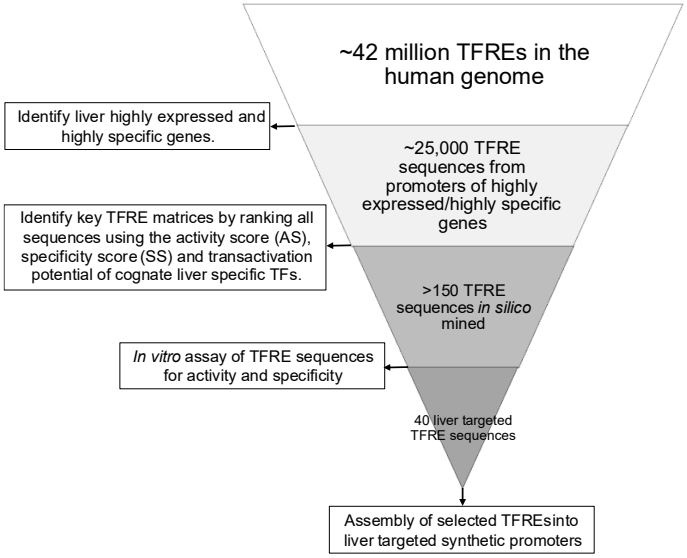

Figure 8. *A schematic showing the workflow used to mine TFREs for the design of liver targeted synthetic promoters.*

References

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SynGenSys Ltd.
5th Floor, 3 St. Paul's Place,
129 Norfolk Street,
Sheffield, S1 2JE
United Kingdom
www.syngensys.com