**High Throughput Screen for the Improvement of Inducible Promoters for Tumor Microenvironment Cues**

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**Abstract**

Cancer immunotherapies are highly potent and are gaining wide clinical usage. However, severe side effects require focusing effector immune cell activities on the tumor microenvironment (TME). We recently developed a chimeric antigen receptor tumor-induced vector (CARTIV), a synthetic promoter activated by TME factors. To improve CARTIV functions including background, activation levels and synergism, we screened a library of promoters with variations in key positions. Here, we present a screening method involving turning on/off stimulating TNFα and IFNγ cytokines, followed by sequential cell sorting. Sequencing of enriched promoters identified seventeen candidates, which were cloned and whose activities were then validated, leading to identification of two CARTIVs with lower background and higher induction. We further combined a third hypoxia element with the two-factor CARTIV, demonstrating additional modular improvement. Our study presents a method of fine-tuning synthetic promoters for desired immunotherapy needs.

**Introduction**

Immune cells are programmed to express effector genes when needed. Interestingly, a few genes are transiently induced following activation by external ques ([1](#_ENREF_1)). Spatiotemporal control over gene expression may improve engineered immune cells.

**The need for inducible promoters**

Immune therapies can eliminate cancerous cells within the body ([2](#_ENREF_2)). Effector immune cells that recognize and attack tumor antigens have already gained clinical approval for chimeric antigen receptor T-cells (CAR-T) ([3](#_ENREF_3)). However, the vigorous activities of CAR-T are also a risk factor ([4](#_ENREF_4)). CAR-T respond to even minute amounts of antigens and may cause severe cytokine storm syndrome, requiring additional regulation of the engineered cells ([5](#_ENREF_5)). To reduce life-threatening side effects, CAR-T are being developed with an intrinsic self-destruction system ([6](#_ENREF_6)), or with ON/OFF switches ([7](#_ENREF_7),[8](#_ENREF_8)). Improving the spatiotemporal regulation of CAR-T and other types of engineered immune effectors will create safer applications of such robust tumor-killing cells ([9](#_ENREF_9)).

**Chimeric antigen receptor tumor-induced vector (CARTIV)**

We recently published a novel approach to regulate effector immune cells using promoters that are inducible by the tumor microenvironment (TME) ([10](#_ENREF_10)). CARTIVs are built by combining binding sites that respond to factors present within the TME. Since tumor microenvironments diverge from normal tissues ([11](#_ENREF_11)), we thought to utilize TME factors as inducers for effector immune cells to act against cancerous cells, and spare normal tissues ([10](#_ENREF_10)). This is somewhat recapitulating the endogenous immune system control of inducing activity within inflammatory sites *in vivo*, thus reducing collateral damage to healthy tissues. Our initial CARTIV promoters demonstrated specific responses to some of the major inflammatory cytokines, such as gamma-interferon (IFNγ) and tumor necrosis factor alpha (TNFα), together with hypoxia. Importantly, numerous studies have attempted to develop such tumor-specific promoters, taking different approaches ([12-15](#_ENREF_12)). We developed the CARTIV approach by combining several minimal binding sites and defined spacers, which resulted in nontrivial findings regarding the number of sites and their relative positions in the promoter ([10](#_ENREF_10)). Our CARTIV promoters are rather short, ranging between 200-300 bases only, and modular, to allow further adjustments and specificity to TME factors. Notably, the first study used basic elements that are not necessarily the best binding sites for background expression or maximal fold induction, creating interest in further improvements.

**Rationale for screening promoters with variations on a theme**

Promoters have "canonical" binding sites of transcription factors, allowing the synthetic design of artificial sequences that may have specific gene expression potency ([16](#_ENREF_16)). Even with advanced protein structure prediction ([17](#_ENREF_17)), it is not easy to predict the complex interactions of several factors with DNA and their cumulative induced transcription. Natural promoters have evolved to provide adequate induction of genes, with divergent response time and magnitude. Changes in the sequences of CARTIV promoters will lead to changes in responses to given stimuli. Specifically, changing only several defined key positions within a given promoter may result in a stronger or more specific response, which is of great interest for clinical applications. Optimization of sequences through screening of libraries is well established ([18-20](#_ENREF_18)), with focus on key positions being possible ([21](#_ENREF_21)). For example, a random DNA sequence of 12 bases has a complexity of 4^12 (over 16 million sequences); rational focus on key positions within binding sites may allow manageable permutations for even a 100-base-long promoter.

**Structure of canonical CARTIV promoter elements**

The original CARTIV promoters ([10](#_ENREF_10)) are based on canonical binding sites. IFNγ signaling through STAT1 includes binding with IFNγ activation site GAS, consensus sequence TTCCNGGAA ([22](#_ENREF_22)). TNFα signaling through NF-kB binds with p50, consensus sequence GGRRRTTYYC, where R is A or G, and Y is C or T ([23](#_ENREF_23)). The hypoxia response elements may include HIF consensus sequence RCGTG ([24](#_ENREF_24)). We simplified these core sequences and added rationally designed repeats and spacers ([10](#_ENREF_10)). Notably, we already achieved nontrivial results regarding the order by which basic elements were assembled when combining hypoxia plus GAS plus kB elements ([10](#_ENREF_10)). Improving CARTIV, or any inducible promoter, requires screening of focused libraries by functional selection for the desired phenotype. CARTIV promoters already show a good response to TME, but may benefit from additional improvements for specific expression levels.

In this study, we aimed to improve CARTIV promoters by functional screening of variations on a theme. Improved promoters may provde nontrivial properties such as robust induction, low background or synergism. First, we focused on critical nucleotides predicted to allow for variations without losing transcription-factor binding. We constructed libraries limited to less than 10^5 complexity, transduced cells in culture and screened them through subsequent rounds of sorting with and without activating cytokine. Sequencing idnetified multiple enriched variants of interest. We further validated some 15 candidates and found improved properties over the original CARTIV promoters. Importantly, we noted that exceptionally high induction or very low background may require different promoter sequences. We also demonstrated that variable nucleotides may have both direct and indirect interactions with their cognate transcription factors. Taken together, we present a method for the functional screening of hundreds of thousands of variant promoters and identification of improved functionalities.

**Methods and Materials**

**Library cloning**

See Supplementary Table 1 for sequences. Oligos, 4 nmol each, were ordered from Integrated DNA Technologies (Coralville, IA). Each library was amplified (15 cycles) using PrimeStar© max (Takara Bio, CA)with primers containing SpeI and MluI restriction enzyme sites. DNA fragments were resolved on agarose gels, extracted, digested and cloned into pHAGE2 plasmids. Plasmids were electroporated into NEB 10-beta cells that were grown overnight in 300 ml LB, and then extracted using a midiprep kit (Macherey-Nagel, Duren, Germany).

**Tissue culture and cytokines**

HEK293T cells were grown in DMEM containing 10% serum, Pen-Strep, HEPES, L-glutamine, non-essential amino acids and sodium pyruvate (Biological Industries, Beit Haemek, Israel). Cells were grown at 37°C in a humidified 5% CO2 incubator. Human recombinant IFNγ and TNFα with activities of 2\*107 Units/mg were purchased from PeproTech (Rehovot, Israel). Standard LV production was as reported ([10](#_ENREF_10)).

**CARTIV promoter activity assay**

HEK293T cells were plated at 1\*105 cells per well in 96-well flat-bottomed plates. Cytokines were added to final concentrations of 500 U/ml. In experiments involving hypoxia, cells were cultured in a hypoxic chamber with a gas mixture of 5% CO2, 0.3% O2 and 94.7% N2 at 20 L/min for 3–5 min, and then sealed and placed at 37°C for 16–20 h before analysis. Cells were harvested, suspended in DAPI 1 µg/mL and measured by FACS using a Beckman Coulter® Gallios™ flow cytometer. Data were analyzed using Kaluza™ software.

**CARTIV library sorting**

HEK293T cells were plated at 3\*106/10 cm plate. Cells were transduced the following day at an MOI of <0.3 (calibrated to have fewer than 30% positive cells to minimize double transduction). Cells were harvested and re-plated for activation at least 72 h post-transduction. Cells were sorted by fluorescent-reporter expression using a FACS aria III (BD) cell sorter for each of the three rounds of selection.

**Library sequencing**

Genomic DNA was extracted and promoters were amplified by PCR for 22 cycles and sent for next-generation sequencing (NGS) at HyLabs (Rehovot, IL). The average number of sequences per sample was 22,368.

**Potency index**

Potency was calculated using the equation *,* where ‘x’ is the normalized GeoMean, ‘G’ and ‘K’ are IFNγ and TNFα treatments, respectively, and ‘bg’ is background GeoMean. When no G or K present, the GeoMean is of cells expressing miniTK alone.

**Statistics**

FACS data are shown as mean ± SD. Data are representative of at least three independent experiments unless otherwise noted. A two-tailed T-test was performed, with p<0.05 considered significant.

**Results**

**CARTIV promoter library**

CARTIV promoters were initially designed by combining multiple binding motifs and linkers ([10](#_ENREF_10)), but were not optimized. In order to improve functional parameters of CARTIV promoters, such as reduced background, increased induction and synergism, we designed a library based on CARTIV promoter G1K0.6 (Figure 1). CARTIV promoter response elements (CPREs) for IFNγ (GCPRE) and TNFα (KCPRE) were adapted from published binding motifs ([22](#_ENREF_22),[23](#_ENREF_23),[25](#_ENREF_25)). A library with 16 variable positions provided a reasonable complexity of 65,536 sequences. For the GCPRE element, we employed the YTTCCSGGAAR sequence (where Y = C/T, S = C/G, and R = A/G). The primary design and the 3'-positions were selected based on the IFNγ-PRE consensus ([26](#_ENREF_26)). We introduced a tandem repeat of this core into the template, separated by a linker AGGGTGGGCAAGT (Figure 1). For the KCPRE element, we employed the GGRRRTTYYC core ([22](#_ENREF_22),[23](#_ENREF_23),[25](#_ENREF_25)) separated by a linker GGGGACTTTCC (Figure 1). DNA oligos with libraries also included a minimal herpes virus thymidine kinase32 (mini TK) ([27](#_ENREF_27)). We amplified and cloned them into a lentivector, as before ([10](#_ENREF_10)), producing libraries of CARTIV promoters with variable key nucleotides.

**Functional screening resulted in improved CARTIV properties**

In order to test whether we could find improved sequences, we screened the libraries by functional activation and sorting (Figure 2A). Three days after transfection of the HEK293 cells with library LVs, we supplemented cell medium with IFNγ and TNFα, and sorted the fraction of responsive cells forty-eight hours later (Figure 2B). The fraction of positive cells was kept low to avoid multiple integration into single cells. Sorted cells were grown for at least 72 hours without cytokines, expanded and sorted for the fraction of cells showing reduced expression (Figure 2B). Notably, a substantial fraction continued to show high expression, suggesting an undesired slow OFF rate. We collected both low-expressing cells and those returning to background levels. We repeated this ON/OFF sorting twice more (Figure 2B), resulting in substantially more positively-induced cells and better decrease rates than primary populations. Finally, we split the double-sorted cells for growth with or without cytokines. In the last sort, we collected six populations according to "low", "medium" and "high" expression from the activated and non-activated groups (Figure 2C), identifying cells with desired ON/OFF switching and coupled controls. Integrated CARTIVs were PCR-amplified and sequenced.

To count how many times each sequence appeared in each treatment, we used the DADA2 algorithm to distinguish single base resolution ([28](#_ENREF_28)). Counts resulted in a total of 497 unique enriched sequences from the library. Sequences with fewer than 150 reads from all six samples were excluded, leaving 161 enriched candidates. Sequences were ranked according to their abundance. We chose sequences that counted fewer reads in the negative fraction and more reads in the medium and high fractions of the activated samples, or an inverse trend in the non-activated samples (Figure 2D). Following this logic, we selected 17 top-ranked candidates (Table 2). Thus, functional screening of the CARTIV library identified a manageable list of variants.

**Enriched sequences reveal variability of key nucleotides**

Next, we wanted to check if any position held profound bias for specific nucleotides. Surprisingly, when examining the selected sequences by multiple sequence alignment, no single variable position was "locked" to a specific nucleotide. In the GAS element, the ratio of the variable positions ranged from 0.36 to 0.52 and averaged 0.43, while in the kappa element, variable bases ranged from 0.22 to 0.5 and averaged 0.35 (Figure 3). The lack of profound bias suggests a high degree of freedom for these positions. In order to better realize the significance of the identified variable nucleotides, we examined the crystal structures of STAT1 (PDB 1BF5) or P50 (PDB 1SVC) with the relevant motif sequences (Figure 4). In both STAT1 and P50 we noted that the protein-DNA interactions are achieved mostly through the DNA phosphate backbone, and not directly over the nitrogenous base of the variable bases (Figure 4). Hence, structural analysis supports the high degree of freedom found in these specific nucleotides, suggesting no strict exclusions but rather good impartiality, allowing fine-tuning of promoters by variation.

**Nontrivial functionalities of selected promoters**

To test for the functional improvement of identified enriched sequences, we aimed for independent validation. All 15 selected variants were synthesized *de novo*, cloned, produced individually in lentiviruses and transduced separately into fresh HEK293 cells. After expansion, cells were split into separate wells for activation by either IFNγ or TNFα, or a combination of both (500 U/ml). FACS analysis measured induction of the fluorescent reporter (Figure 5A, and Supplementary Figures 1 and 2). Notably, little response was elicited by IFNγ alone, while most sequences showed significant response to TNFα and even higher induction by a combination of IFNγ and TNFα (Figure 5B). Sequences 79 and 143 exhibited the highest overall response by simple gross analysis, while sequences 4, 5, 11 and 130 also showed a substantial induction (SupplementaryFigures 2 and 3). Analysis normalizing reporter expression to the no-cytokine baseline showed that sequences 5 and 130 had the highest specific response (7.66- and 11.92-fold increase, respectively). Seq 5 and Seq 130 also showed the highest specific response to TNFα alone (4.12- and 3.83-fold increase, respectively). Intriguingly, IFNγ did not elicit a very strong response by itself, but showed major contribution when coupled with TNFα. Thus, we wanted to find out whether the effect of cytokine combination was additive or synergistic. We divided the response of the promoter to the combination of cytokiness with the sum of responses to IFNγ and to TNFα (MFIgk/ (MFIg+MFIk)). By this analysis, the combination of cytokines had the most synergistic effect in sequences 5 and 130 (1.46 and 2.28, respectively, Figure 5C). Out of the 15 enriched sequences, we found independent validation for improved induction and synergism, with two leading hits for further use.

An important parameter for CARTIV promoters is their background level, i.e., expression without cytokines. To determine background, we measured the fluorescence levels of cells transduced with vectors having the miniTK promoter only, and compared them with the selected CARTIVs without cytokines. Data indicated that sequences 90 and 130 had the lowest background (1.04 and 1.63, respectively). In contrast, sequences 11, 79 and 143 had the highest background (5.24, 12.38 and 15.29). In addition, other variants showed low background, among them sequence 5 (Figure 5d and Supplementary Figures 3 and 4). This suggests that variations of key nucleotides may provide different levels of background, or basal leakiness.

In order to logically score the overall functionality of the selected promoters, we calculated a "potency index" (see Methods), taking into account activation levels, background levels and synergism. In accordance with above-mentioned activation data, Seq 130 and Seq 5 appeared to be the most potent (11.38 and 6.96 scores, respectively). In contrast, some promoters achieved a score close to zero, including the controls, miniTK and the ef1α promoters (Figure 5D). Thus, promoters achieving high potency scores have high activation levels and high synergism levels between their promoter response elements (PREs) and low background levels. This index further suggests nontrivial improvements following screening through random changes of nucleotides within CARTIV promoters.

Next, we wanted to test the time it takes to turn the new variant promotors on and off. The leading hits, Seq 5 and Seq 130, were tested for their kinetics. Transduced HEK293 cells were activated by IFNγ and TNFα (500 U/mL each) and tracked using a lionheart fx automated microscope. Seq 5 showed a faster ON rate (Figure 6A).Next, we withdrew cytokines and followed the cells. As seen in Figure 6B, the “OFF” rate was very similar for Seq 5 and Seq 130. Taken together,variations of key nucleotides change not only expression levels, but also the relative kinetics of induction.

**Additional hypoxia PRE can further enhance activity of selected promoters**

In a previous study, we investigated the contribution of hypoxia PRE (HCPRE) by adding it to CARTIV promoters ([10](#_ENREF_10)). Since Seq 130 and Seq 5 had the best potency scores of the G1K06 variants tested, we sought to combine them with HCPRE. Promoters HG1K06-130 (H130) and HG1K06-5 (H5) were cloned and transduced into fresh cells. After expansion, the cells were tested without cytokines, with IFNγ, with TNFα, or with both, and subjected to hypoxic conditions for the last 18 hours before reading signal intensity. In agreement with previous experiments, the combination of IFNγ and TNFα resulted in high induction (Figure 7B, left panel). The hypoxia HCPRE further enhanced the reporter levels. By the raw data, H5 and H130 showed similar enhanced expression with the HCPRE (Figure 7B, left). Analyzing for specific expression (normalizing to background) revealed that H130 had higher-fold induction than H5 (Figure 7B, right). Hence, the modularity of CARTIV promoters is sustained with improved sequences, allowing for further addition of response elements that show higher levels of expression. Our improved CARTIV promoters retain the modularity and possible combination with additional PRE for more TME factors.

**Discussion**

**Focus on the tumor**

In this study, we functionally screened *de novo* variations of synthetic promoters. A series of FACS sorting with and without stimulation (ON and OFF states) identified numerous candidates that were validated independently, revealing nontrivial combinatorial effects of key nucleotides. We demonstrate improvements including low background and high fold activation in response to two factors characteristic of TME. Moreover, we show that these improved promoters can further benefit from the addition of a third element, demonstrating modularity of CARTIV design.

**CARTIV elements – the basis for variations**

Our first line of CARTIV showed good induction by a combination of TME factors, such as TNFα, IFNγ and hypoxia ([10](#_ENREF_10)); however, activation by IFNγ alone was relatively low. The basic GAS PRE element has the typical sequence for STAT binding ([22](#_ENREF_22)). Variations of selected nucleotides (Figure 3-4) showed a modest increase of IFNγ alone, and a more substantial synergistic induction together with TNFα. These data suggest some freedom of these nucleotides, not abrogating the interactions with STAT proteins but rather fine-tuning the CARTIV activity. Importantly, in this study we focused on changes within binding sites, while the synergistic activities may be further modified according to the linkers, opening the opportunity for additional improvements.

The STAT proteins contact the GAS DNA sequences in a 15-bp region ([22](#_ENREF_22),[29](#_ENREF_29),[30](#_ENREF_30)). According to Chen *et al.* ([30](#_ENREF_30)), the optimal DNA binding for STAT1 was suggested as AHTTCCSGGAAD (or explicitly, A[A/C/T] TTCC[C/G]GGAA[G/A/T]TG). For library construction we used AYTTCCSGGAARTG (Figure 1). In our 15 selected promoters we saw no profound bias towards a single nucleotide at any of the variable positons (Figure 3). Importantly, Seq 130 that showed the best synergism results from having only 2-base difference from Seq 5, and no obvious overall difference from all other 15 CARTIV promoters (Figure 3). Therefore, our data suggest a nontrivial effect of specific nucleotide variations on the overall activity, and the synergistic effect of the GAS portion with the CARTIV promoter.

The CARTIV kappa element was based on the sequence GGRRRTTYYC ([23](#_ENREF_23)). Chen *et al.* published a similar core sequence of GGGRNWTTCC ([31](#_ENREF_31)). In our 15 sequences, we found sequences starting with GGGG that were associated with p50, and others starting with GGAA that were associated with RELA ([23](#_ENREF_23)). Interestingly, Seq 130 has GGGGG in the first kappa element and GGGAA in the second. This suggests binding of RELA-p50 to the first site and RELA-RELA to the second site. On the other hand, Seq 5 has GGGAG and GGGGA, suggesting that both may bias for RELA-p50 and not for RELA-RELA. Activation by TNFα only was similar between Seq 5 and Seq 130, but synergism was better with Seq 130, possibly due to the heterogenous usage of the binding dimers. Our data suggest that kappa PRE may respond promiscuously to p50 and/or RELA.

**Nontrivial functions of selected CARTIV promoters**

We have previousy shown that it is possible to increase expression levels by increasing repeats of elements (e.g., G1K1<G2K2<G3K3). However, this may cost substantial background expression and reduction in synergism ([10](#_ENREF_10)). One of the major limitations of CAR-T treatments in solid tumors is the “on target, off tumor” toxicity ([4](#_ENREF_4),[32](#_ENREF_32),[33](#_ENREF_33)). Here, a library screen identified variants with nontrivial reduced background and increased synergism (Figure 5). This may provide for sufficient CAR expression only at the TME, sparing normal healthy tissues. Variations on the theme of modular CARTIV promoters offer fine tuning of CAR or other immunotherapies where spatiotemporal control is needed ([9](#_ENREF_9),[34](#_ENREF_34)).

**Structural consideration of variable bases**

The selected variable positions are within the DNA-protein interactions, according to structural data (Figure 4). The high degree of freedom noted in these positions (Figure 3) implies that when designing a promotor sequence, it is advisable to examine all positions that interact or are predicted to interact with the protein by the phosphate backbone rather than the nitrogenous base. Fang *et al.* showed that a single SNP in a DNA binding recognition site can influence transcription factor binding, thereby affecting gene regulation ([35](#_ENREF_35)). Thus, our engineering of synthetic promoters is also relevant for natural variations among humans. One may assume low or no tolerance for variability where a nitrogenous base interacts directly with a transcription factor. Interestingly, Le *et al.* demonstrated that dinucleotides flanking the core promoter sequence can contribute significantly to transcription factor binding ([36](#_ENREF_36)), thus adding fine tuning of activities. Nevertheless, as we have demonstrated, a wide range of nontrivial properties can be observed when screening the variable positions of a known consensus sequence, with additional complexity when using multiple binding sites.

**The advantages of multiple PRE combinations**

Our library was based on our basic CARTIV promoter designed to regulate effector gene expression within the TME ([10](#_ENREF_10)), thanks to the abundance of inflammatory cytokines such as TNFα and IFNγ (36). TNFα and IFNγ might also be present in inflammatory sites other than TME. Therefore, additional PREs that correspond to TME and not to inflammatory sites will help ([37](#_ENREF_37)). Hypoxia is a hallmark of TME ([38](#_ENREF_38)), and we demonstrated the possible addition of an HCPRE to CARTIV (11). The cumulative synergistic improvement of HCPRE with Seq 5 or Seq 130 (Figure 7) suggests that even improved CARTIVs sustain modularity and optional enhancement by an additional third party.

**Conclusion**

We present an approach to designing, screening and functionally validating inducible promoters with different traits that may improve engineered immune cells. The dogma of “one treatment fits all” is shifting towards precision treatment ([39](#_ENREF_39)). CARTIV promoters with variations and modular modifications can provide engineered immune cells for specific TMEs.

**5. Data Availability**

**6. Funding**

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**7. Conflict of interest**

The authors declare no conflicts of interest.

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**Figure Legends**

**Figure 1 – The G1K06 library design.** The basic structure of the G1K06 CARTIV promoter and the variable elements used to construct the CARTIV promoter library. Red arrows indicate variable nucleotide position. Y=C/T, S=G/C, R=A/G.

**Figure 2 – The GK CARTIV promoter library showing increments for responsive promoters following three sorting rounds.** (**A**) An overview of the steps applied to the library screen. (**B**) FACS plots of HEK293T cells infected using lentiviruses with ZsGreen under the control of the CARTIV promoter library, showing three consecutive rounds of activation and relaxation. Red boxes and arrows show the sorted population in the specific selection round, 72 hours post-infection. Cells were treated with 500 U/mL of IFNγ and TNFα for 48 hours, then harvested and processed for cell sorting. Data are single-discriminated. (**C**) FACS plots of HEK293T cells after three consecutive rounds of positive and negative selection, showing data and gating for the six-cell population extracted for sequencing and frequency cross-analysis. Data are single-discriminated and DAPI-negative. (**D**) Frequency cross analysis for six representative promotors selected for the functional studies. Cut-off for reading number was 150; only sequences showing “x” trend were selected for cloning.

**Figure 3 – The selected GK CARTIV promoter library shows no strong tendency for a specific position**. (**A**) Multiple sequence alignment of GAS elements. (**B**) MSA of KB elements. Each row represents a sequence derived from the bioinformatics analysis. A distribution analysis between 0 to 1 is shown below A and B.

**Figure 4 – DNA in the variable positions in the CARTIV library interact with P50 and STAT1 by the phosphate backbone structures of STAT1 and P50 crystalized with a consensus DNA sequence.** Left panel: the crystal structure of STAT1 together with STAT1 binding sequence, in orange the 5‘ DNA strand, in dark green the 3‘ DNA strand. In bright green, the nucleotide interacting with the protein, marked on the DNA sequence, is the relevant nucleotide pair, indicating the interacting nucleotide. The right panel shows the crystal structure of P50 with a binding sequence; the 5‘ DNA strand is shown in dark green. The nucleotide interacting with the protein marking the relevant nucleotide pair on the DNA sequence and indicating the interacting nucleotide is shown in bright green.

**Figure 5 – Promoters 5 and 130 show a robust and synergistic following IFN and TNF activation.** (**A**) Representative FACS plots from a. (seq 130). Cells were single-discriminated and gated on Dapi- (**B**) Normalized reporter expression. Each clone was treated with IFNγ and TNFα. The reporter’s geometric mean fluorescent intensity of each clone was divided by the response of the non-treated of the same clone. (**C**) Synergism was calculated by dividing the geometric mean of the fluorescence intensity (MFI) of cells stimulated with IFNγ and TNFα divided by the sum MFI of cells stimulated with IFNγ or TNFα vs. background levels (solid gray bars) were calculated by dividing the MFI of transduced cells by the MFI of non-infected cells in the same well. (**D**) Potency score (dashed bars). Data from one of two experiments are shown.

**Figure 6 – Sequence 5 displays better activation kinetics.** (**A**) Activation rate. Y-axis: normalized RFP670 expression; X-axis: time. Time interval is 1 hour. (**B**) Deactivation rate. Medium was replaced with fresh medium without cytokines at time zero.

**Figure 7 – Adding complexity to the G1K06-5/130 promoters does not affect response to external stimuli.** (**A**) The basic structure of the HG1K06 CARTIV promoters. (**B**) The geometric mean of RFP670 in ZsGreen-positive cells, showing the average of triplicates. Error bars indicate standard deviation.

**Supplementary Figure 1.** Representative FACS plots of selected library sequences displaying promoter expression in response to cytokine combination. Data shown are single-discriminated, and Dapi-.

**Supplementary Figure 2.** G1K06-library-derived sequence, raw GeoMFI.

**Supplementary Figure 3.** Background levels. Clone background MFI levels were divided by miniTK MFI.

**Supplementary Figure 4.** Potency score calculated for raw GeoMFI.