**High throughput screen for the improvement of inducible-promoters for tumor-microenvironment cues**

Omri Sharabi, Yariv Greenshpan, Noa Ofir, Aner Otholongi, Leonid Olender, Zachor Adler-Agmon , Angel Porgador, Roi Gazit\*

The Shraga Segal Department of Microbiology, Immunology, and Genetics, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer Sheva, 8410501, Israel

\*Corresponding: Roi Gazit gazitroi@bgu.ac.il

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**Abstract**:

Cancer immunotherapies are highly potent, gaining wide clinical usage. However, severe side effects require the focus of effector immune cells activities on the tumor microenvironment (TME). We recently developed Chimeric Antigen Receptor Tumor-Induced Vector (CARTIV), synthetic promoters activated by TME factors. To improve CARTIV functions, such as background, activation levels, or synergism, we screen a library of promoters with variations of key positions. We present the method to screen the promoter's library through sequential sorting of cells after turning ON/OFF the stimulating TNFα and IFNγ cytokines. Sequencing of enriched-promoters identified seventeen candidates. We cloned each and validated activities, finding two CARTIVs with lower background and higher induction. We further combine a third hypoxia element with the two factors CARTIV, demonstrating additional modular improvement. Our study presents the method to fine-tune synthetic promoters for desired immunotherapies needs.

1. **Introduction:**

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

**1.1 The need for inducible promoters**

Immune-therapies are eliminating cancerous cells within the body ([2](#_ENREF_2)). Effector immune cells that recognize and attack tumor-antigens already gained clinical approval for Chimeric-Antigen-Receptor T-cell (CAR-T) ([3](#_ENREF_3)). However, the vigorous activities of CAR-T are also a risk factor ([4](#_ENREF_4)). CAR-T do respond even to minute amounts of antigens and might cause severe cytokine-storm syndrome, requesting 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 ([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)).

* 1. **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 combinations of binding sites that respond to factors present within the TME. Tumors microenvironment diverges from normal tissues ([11](#_ENREF_11)), we thought to utilize TME-factors as inducer for effector immune cells to focus 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 of 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 had attempted to gain such tumor-specific promoters, taking different approaches ([12-15](#_ENREF_12)). We brought the CARTIV approach by combining several minimal-binding-sites and defined spacers, which gained nontrivial findings regarding the number of sites and their relative positions in the promoter ([10](#_ENREF_10)). Our CARTIV promoters are rather short, ranging 200-300 base 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 in background expression or maximal fold induction, creating an interest in further improvements.

* 1. **Rationale for screening variations of a theme promoters**

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 had evolved to provide adequate induction of genes, with divergent response time and magnitude. Changes in the sequences of CARTIV promoter will provide changes of response to given stimuli. Specifically, changing only several defined key positions within a given promoter may gain stronger or more specific response, which is of great interest for the clinical applications. Optimization of sequences through screening of libraries is well-established ([18-20](#_ENREF_18)), with the possible focus on key positions ([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 even for a 100 bases long promoter.

* 1. **Structure of canonical CARTIV promoter elements**

The original CARTIV promoters([10](#_ENREF_10)) are based on canonical binding sites. IFNγ signaling through STAT1 includes the binding with IFNγ activation site GAS, consensus sequence: TTCCNGGAA ([22](#_ENREF_22)). TNFα signaling through NF-kB is binding 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 had simplified these core sequences and added rationally designed repeats and spacers ([10](#_ENREF_10)). Notably, we already gained 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 gain additional improvements for specific needs of expression levels.

In this study, we aimed to improve CARTIV promoters by functional screening of variations over a theme. Improved promoters may pose nontrivial properties such as robust induction, low background, or synergism. First, we focused on critical nucleotides predicted to allow for variations without losing transcription-factors binding. We constructed libraries limited to less than 10^5 complexity, transduced cells in culture, and screened them through consequent rounds of sorting with and w/o activating-cytokine. Then, sequencing revealed multiple enriched variants of interest. We further validated some 15 candidates and found improved properties over the original CARTIV promoters. Importantly, we note that exceptional-high induction or very-low background may require different promoter sequences. We also demonstrate that the variable nucleotide may have direct and indirect interactions with their cognate transcription factors. Taken together, we present a method for the functional screen through hundred-thousand variant promoters and identify improved functionalities.

**2. Methods**

*2.1 Library cloning*

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

*2.2 Tissue culture and cytokines*

HEK293T 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 were purchased from PeproTech (Rehovot, Israel); with activity of 2\*107 Units/mg. Standard LV production was as reported ([10](#_ENREF_10)).

*2.3 CARTIV promoter activity assay*

HEK293T cells were plated at 1\*105 per well in a 96 plate flat bottom. Cytokines were added to final concentrations of 500 U/ml. In experiments involving Hypoxia, cells were cultured in an hypoxic chamber, with 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 with DAPI 1 µg/mL and FACS measured using Beckman Coulter® Gallios™ flow cytometer. Data were analyzed using Kaluza™ software.

*2.4 CARTIV library sorting*

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

*2.5 Library sequencing*

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

*2.6 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.

*2.7 Statistics*

FACS data are shown as means ± SD. Data are representative of at least three independent experiments unless otherwise noted. T-test (two tales) was performed considering p < 0.05 as significant.

**3. Results**

**3.1 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 (CPRE’s) for IFNγ (GCPRE) and TNFα (KCPRE) are adapted from published binding motifs ([22](#_ENREF_22),[23](#_ENREF_23),[25](#_ENREF_25)). A library with 16 variable positions provided 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)), gaining libraries of CARTIV promoters with variable key nucleotides.

**3.2 Functional screen gain improved CARTIV properties**

In order to test whether we can 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 cells medium with IFNγ and TNFα. Forty-eight hours later we sorted the fraction of responsive cells (Figure 2B). The fraction of positive cells was low to avoid multiple integration into single cells. Sorted cells were grown for at least 72 hours w/o cytokines, expanded, and sorted for the fraction of cells showing reduced expression (Figure 2B). Notably, a substantial fraction kept high expression, suggesting for 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), gaining substantially more positively-induced cells and better decrease rates than primary populations. Finally, we split the double-sorted cells for growth with or w/o cytokines. In the last sort, we collected six populations according to "low", "medium" and "high" expression from the activated and from the non-activated groups (Figure 2C), gaining 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 a single-base resolution ([28](#_ENREF_28)). Count resulted in a total of 497 unique enriched sequences from the library. Sequences with less than 150 reads from all 6 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 times 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). The functional screen of CARTIV library identified a manageable list of variants.

**3.3 Enriched sequences reveal variability of key nucleotides**

Next we wanted to check if any position had gained profound bias for specific nucleotide. Surprisingly, when examining the selected sequences by multiple sequences 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 at 0.43 while in the Kappa element variable bases ranged from 0.22 to 0.5 and averaged at 0.35 (Figure 3). The lack of profound bias suggests for high degree of freedom for these positions. In order to better realize the significance of the identified variable nucleotide, we examined the crystal structures of STAT1 (PDB 1BF5) or P50 (PDB 1SVC) with the relevant motifs sequences (Figure 4). In both the STAT1 and the P50 we noted that the interactions between the protein and the DNA is 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 variations.

**3.4 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, individually produced in lentiviruses and transduced separately into fresh HEK293 cells. After expansion, cells were split to separate wells for activation by either IFNγ, or TNFα, or the combination of both (500 U/ml). FACS analysis measured the induction of the fluorescent reporter (Figure 5A, and supplementary Figure 1 and 2). Notably, only little response was elicited by IFNγ alone, while most sequences showed significant response to TNFα and even higher induction by the combination of IFNγ with 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 base-line found 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 cytokines 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 by 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 with the selected CARTIVs w/o cytokines. Data indicated that Seq 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 Figure 3 and 4). This suggest 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 abovementioned 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). Therefore, promoters achieving high potency score have high activation, high synergism levels between their PRE’s and low background levels. This index further suggest for nontrivial improvements following screen through random changes of nucleotides within CARTIV promoters.

Next we wanted to test for the time it takes to turn our new variant-promotors ON and OFF. The leading hits Seq 5 and Seq 130 were tested for their kinetic. 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 can be seen in Figure 6B, the “OFF” rate was very similar for Seq 5 and Seq 130. Taken together,variations of key-nucleotides not only change expression levels, but also the relative kinetics of induction.

**3.5 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 the 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 w/o cytokines, with IFNγ, with TNFα or 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 gained similar enhanced expression with the HCPRE (Figure 7B, left). Analyzing for the specific expression (normalizing to background) found that H130 gained 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 gain higher levels of expression. Our improved CARTIV promoters retain the modularity and possible combination with additional PRE for more TME factors.

**4. Discussion**

**4.1 Focus on the tumor**

In this study we functionally screened *de novo* variations of synthetic promoters. A series of FACS sorting with and w/o stimulation (ON and OFF states) gained 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 these improved promoters can further benefit from the addition of a third element demonstrating modularity of CARTIV design.

**4.2 CARTIV elements – the basis for variations**

Our first line of CARTIV gained good induction by a combination of TME factors, such as TNFα, IFNγ, and Hypoxia ([10](#_ENREF_10)); however, the 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) gained a modest increase of IFNγ alone, and a more substantial synergistic induction together with TNFα. These data suggest for 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, which opens 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 see no profound bias towards a single nucleotide at any of the variable positons (Figure 3). Importantly, Seq 130 that showed the best synergism is having only 2-base difference of 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 similar core sequence of GGGRNWTTCC ([31](#_ENREF_31)). In our 15 sequences, we find sequences starting with GGGG that was associated with p50, and others starting with GGAA that was associated with RELA ([23](#_ENREF_23)). Interestingly, Seq 130 has GGGGG in the first kappa element and GGGAA in the second. This may suggest the binding of RELA-p50 to the first and RELA-RELA to the second site. On the other hand, Seq 5 is having GGGAG, and GGGGA suggesting both may bias for RELA-p50 and not for RELA-RELA. The activation by TNFα only was similar between Seq 5 and 130, but synergism was better with Seq 130, possibly by the heterogeneous usage the binding dimmers. Our data suggest that kappa PRE may promiscuously respond to p50 and/or RELA.

**4.3 Nontrivial functions of selected CARTIV promoters**

Previously we have shown that it is possible to increase expression levels by increasing repeats of element (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)). 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 over theme modular CARTIV promoter offer fine-tuning CAR or other immune-therapies where spatiotemporal control is needed ([9](#_ENREF_9),[34](#_ENREF_34)).

**4.4** **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) imply that when designing a promotor sequence, it will be advised 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. have shown that a single SNP in DNA binding recognition site can influence transcription factor binding, thus affecting gene regulation ([35](#_ENREF_35)). Thus, our engineering of synthetic promoter is also relevant for natural variations among humans. One may assume low or no tolerance for variability where nitrogenous base interact 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.

**4.5 The advantages of multiple PREs 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 present in other inflammatory sites out of the TME. Therefore, additional PRE that corresponds 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.

**4.6 Conclusion**

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

**5. Data Availability**

**6. Funding**

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**7. Conflict of interest**

The authors declare no conflict of interests

**7. References**

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**Figure legeneds**

**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 show increment 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. They are showing three consecutive rounds of activation and relaxation. Red boxes and arrows show the sorted population in the specific selection round; 72 hours following infection, cells were treated with 500U/mL of IFNγ and TNFα for 48 hours, then harvested and proceeded for cell sorting. Data is single discriminated (**C**) FACS plots HEK293T cells after three consecutive rounds of positive and negative selection. They are showing data and gating for the six-cell population extracted for sequencing and frequency cross-analysis. Data is 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. At the bottom of A and B is a distribution analysis between 0 to 1.

**Figure 4. The 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 deep green the 3‘ DNA strand. In glowing green, the nucleotide interacting with the protein, marked on the DNA sequence is the relevant nucleotide pair, outlined the interacting nucleotide. The right panel showing the crystal structure of P50 with a binding sequence; in deep green, the 5‘ DNA strand. In glowing green, the nucleotide interacting with the protein marked the relevant nucleotide pair on the DNA sequence outlined the interacting nucleotide.

**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 IFNg and TNFa. 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 is calculated by dividing the geometric mean of the fluorescence intensity (MFI) of cells stimulated with IFNg and TNFa divided by the sum MFI of cells stimulated with IFNg or TNFa vs. background levels (solid grey 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 are shown from one out of 2 experiments.

**Figure 6. Sequence 5 displays better activation kinetic.** (**A**) activation rate. The Y-axis is normalized RFP670 expression. The X-axis is time. The time interval is 1 hour. (**B**) deactivation rate. Media was replaced with fresh media without cytokines at time zero.

**Figure 7. Adding complexity to the G1K06-5/130 promoters does not effect 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 indicated 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 sequences raw GeoMFI

**Supplementary figure 3** background levels. Clones background MFI levels were divided by miniTK MFI

**Supplementary Figure 4** potency score calculated for raw GeoMFI