BBF RFC 42: RA-PCR, a method for the generation of randomized promoter libraries

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17th of October 2009

1. Purpose

The purpose of this RFC is to provide instructions for the synthesis of promoters in mammalian cells that are active at a desired cellular condition (where a cellular condition is specified by the activity of a set of transcription factors of interest). The method generates a library of promoters putatively active under the desired condition. This RFC also provides instructions on how to screen the libraries generated by this method in order to obtain functional promoters. Description of promoters generated by this method can be found at http://2009.igem.org/Team:Heidelberg/Project_Synthetic_promoters.

2. Relation to other BBF RFCs

This RFC (Request for Comments) is a new proposal for standardized promoter synthesis. It describes a method for synthesis that is unrelated to existing RFCs. Promoters synthesized by the method described in this RFC are compatible with all existing standards.

3. Copyright Notice

4. Introduction to RA-PCR

Random Assembly PCR / RA-PCR is a standard method for the construction of randomized promoter libraries. It is modified from Assembly PCR [1] to create promoters with randomized spacing and frequency of Transcription Factor Binding Sites. It relies on using different oligos containing a transcription factor binding site (or random DNA) plus two annealing sequences (see Figure 1 for a comprehensive explanation of the method). It uses two sets of oligos, one for the top strand, and one for the bottom strand. The oligos for each strand have the same two annealing sequences (which are complementary to the annealing sequences of the other strand). If these oligos are pooled, they will randomly anneal to each other, thus generating randomized repeats of the transcription factor binding sites of interest at varying spacing. In order to be able to clone the construct, two stop oligos (termed stop 5' and top 3') which contain only one annealing sequence, plus a cutsite (SpeI 5' SHOULD be used in order to stay compatible

with RCF-12; Another site SHOULD be used if the promoter is to be constructed to be compatible with another cloning standard. HindIII 3' SHOULD be used) MUST be added. Double-stranded DNA is created by running a seven-cycle PCR, and amplified by a 25-cycle PCR. Then, the resulting (proximal) promoter is cloned 5' of a core promoter. This MAY be done by inserting it into pSMB_MEASURE (Part:BBa_K203100), a promoter measurement / screening plasmid backbone for use in mammalian cells. Thus, a mixture of different promoters in the same plasmid backbone is generated. These can then be transformed into bacteria. Each colony represents a single putative promoter, which can be transfected into mammalian cells under the conditions of interest, plus control conditions. Promoters active under the desired conditions, but not under control conditions, are selected for further characterization.
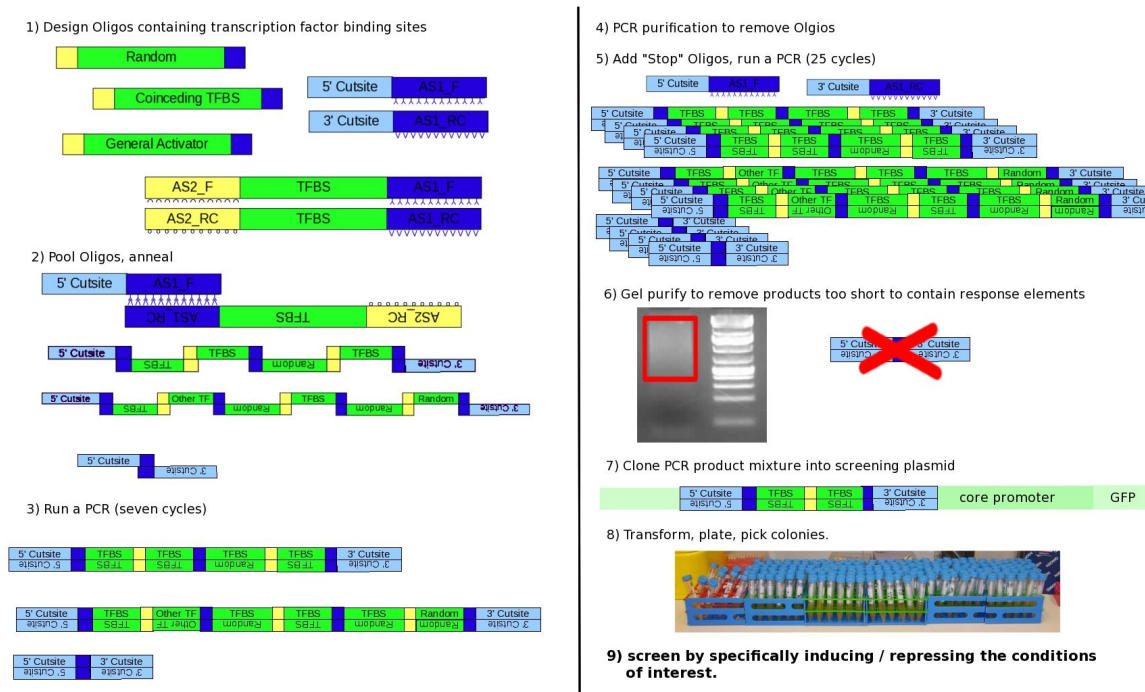

Figure 1: The method of RA-PCR

5. RA-PCR protocol

1. MUST design two annealing sites, each 15-18 base pairs long. Annealing sites MUST be void of transcription factor binding sites, and restriction sites from one of the following enzymes: EcoRI, XbaI, SpeI, PstI, BglII, NgoMIV, NheI and HinDIII. The reverse complement of both sequences MUST be calculated. The following sequences MAY be used.

	Forward (F)	Reverse Complement (RC)
Annealing Sequence 1 (AS1)	GGGTGACGGGTTCA	AGTGAACCCGTCACCC
Annealing Sequence 2 (AS2)	GCGATCGGCAGATCA	TGATCTGCCGATCGC

2. MUST design 5' stop oligos containing (5' to 3') four base pairs (e.g. GATC), a cutsite (SpeI for RFC12 cloning standard) and AS1_F. Appendix 1 contains a list of oligos that MAY be used.
3. MUST design 3' stop oligos containing (5' to 3') four base pairs (e.g. GATC), a cutsite (HindIII MAY be used) and AS1_RC. Appendix 1 contains a list of oligos that MAY be used.
4. MUST design forward and reverse oligos for each transcription factor of interest. Forward oligos contain AS2_F, the transcription factor binding site (TFBS) and AS1_F. Reverse oligos contain AS2_RC, the TFBS and AS1_RC. TFBS SHOULD be designed to represent the matrix describing the factor's binding preferences according to section 7. Appendix 1 contains a list of oligos that MAY be used.
5. MAY design forward and/or reverse oligos for coinceding transcription factors. RFC 43 MAY be used in order to analyze the trancscription in the same way as described in step 6. Appendix 1 contains a list of oligos that MAY be used.
6. MAY design forward and/or reverse oligos for general activators (Sp1, Ap1, CREB and NF-Y). Appendix 1 contains a list of oligos that MAY be used.
7. SHOULD design forward and reverse spacer oligos, which contain 10-15*dNTPs (random nucleotides) instead of a TFBS. Appendix 1 contains a list of oligos that MAY be used.
8. SHOULD order oligos at 100 µM.
9. SHOULD pool the oligos. As a general rule, use 0,8 µL of Stop 5' and Stop 3'; ~4 µL of the transcription factor (forward), ~4 µL of the transcription factor (reverse), 1-2 µL each of the forward and reverse spacer oligo, ~1 µL of coinceding transcription factors and a total of 0,5 µL of general activators. Define Vo as the total Volume of oligos pooled.
10. MUST add Oligos (volume Vo) to water in total volume Vw = (10 * Vo – Vo). Add volume Vo of diluted Oligos Vw to water in volume Vf = (10 * Vo – Vo). Add volume Vo of final oligo dilution Vf to a PCR reaction. Total reaction volume SHOULD be 50 µL
11. MUST run the PCR 7-10 cycles, SHOULD use the following setup:
    1 cycle Initial dentaturing, 5 minute 95 °C
    7-10 cycles assembly: 30 seconds 95 °C, 45 seconds 58 °C, 45 seconds 72 °C
    Terminal hold, 4 °C, forever
12. MUST remove oligonucleotides by performing a PCR purification using PCR purification kit (e.g. QIAGEN) or a gel extraction using Gel extraction kit (e.g. QIAGEN)
13. MUST add PCR reagent again. Add 5' stop oligo and 3' Stop oligo, 25 pmol (1 µL of 1:4 diluted stock).
14. MUST run the PCR, 25 cycles, with the following setup:
    1 cycle Initial dentaturing, 5 minute 95 °C
    25 cycles amplification 30 seconds 95 °C, 45 seconds 68 °C, 60 seconds 72 °C

Terminal hold, 4 °C, forever
15. MUST gel purify PCR products to exclude everything <200Bp. Use a 1% agarose gel, 50 V for at least 2 h to achieve a good resolution
16.   MUST digest with HindIII and SpeI (or whatever cutsites were included in step 4 and 5). Digest a reporter plasmid containing a core promoter and a reporter gene with the same enzymes. MAY use BBa_K203100 for this task. SHOULD perform a thorough digest; in addition, digest the plasmid with shrimp alkaline phosphatase or calf intestine phosphatase afterwards. Gel purify the plasmid backbone, PCR purify the digested PCR products.
17. MUST ligate. Perform a thorough ligation to increase transformation efficiency. We used Fermentas T4 DNA Ligase for 5 h, 19 °C or overnight, 16 °C.
18. MUST transform into competent *E. coli* cells and plate out. Pick no more than 20 colonies per individual PCR reaction. If more putative promoters are desired, set up several PCR reactions
19. Isolate plasmid DNA from the selected colonies. We used QIAGEN Miniprep kit for this tasked.
20. MAY test-digest miniprep DNA with the same enzymes used in step 16 to make sure you get plasmid with synthesized promoters of varying length. Length of the inserts (that is, synthetic promoters) should be between 100 and 600 basepairs. If this is not the case, vary stop oligo concentration in step 9, improve gel purification setup in step 15 or alter PCR conditions in step 11 and 14.
21. MUST perfom a screening to select functioning clones. For example, transfect clones in triplicates into eukaryotic cells on a 96 well plate by using transfection agents such as EFFECTENE (QIAGEN) or Lipofectamine (Invitrogen). Then, induce the conditions of interest in one replicate, shut them off in a second replicate, and leave control medium on the third replicate. When the pathway is fully active, read flourescence (or luminescence, if a luciferase reporter is used) by a plate reader (TECAN) or other automated methods.

6. Improved screening

We experienced difficulties inducing some of the pathways we were trying to synthesize promoters for (namely, HIF and p53). We learned that finding the ideal time point of induction for a certain pathway and the ideal conditions is very difficult even with literature at hand. Therefore we suggest that for future screening, a library of siRNAs for the transcription factors of interest SHOULD be compiled. Also, a library of transcription factors mutated to be constantly active is required. With these libraries at hand, individual transcription factors can be knocked down and activated specifically at 100% efficiency. This will greatly facilitate screening and parts characterization.

7. Oligo design (advanced)

Oligos SHOULD be designed to reflect sequence variation among different Binding sites and not be a mere representation of the consensus sequence of such binding sites, as shown at the example of NF-κB. Considering the graphical representation of NF-κB's

frequency matrix shown in Figure 2 (source: JASPAR, http://jaspar.cgb.ki.se/), the oligo SHOULD be designed in order to represent this matrix, instead of a static NF-κB binding site. A sensible representation of this matrix would be GGGRHTTYCC (for the IUPAC nucleotide code, refer to http://www.bioinformatics.org/sms/iupac.html). Most oligonucleotide manufactures provide the option to synthesize such mixtures of individual oliogs without further cost. RA-PCR is therefore able to synthesize even promoters responsive to badly-described transcription factors
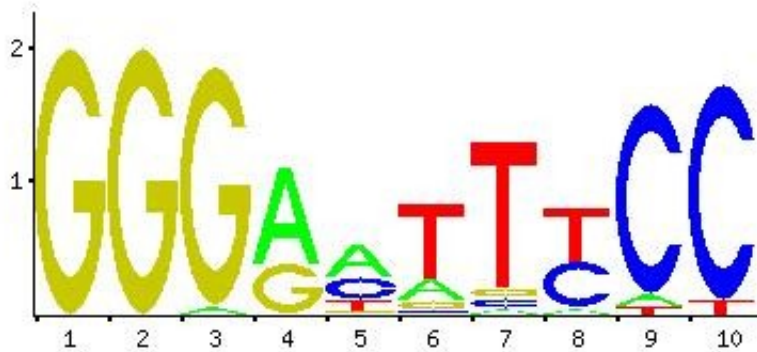

Figure 2: NF-kB frequency matrix

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References

[1] Stemmer, W.P.C. et al. Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides. Gene 164, 49-53 (1995).

Supplementary information can also be found at
http://2009.igem.org/Team:Heidelberg/Project_Synthetic_promoters

Appendix 1: Oligos for use in RA-PCR

Stop 5'	CAGTACTAGTGGGTGACGGGTTCA
Stop 3'	TGACAAGCTTAGTGAACCCGTCACCC
Sp1 responsive (general transactivator)	GCGATCGGCAGATCAGGGGCGGGGCGGGTGACGGGTTCA
Ap1 responsive (general transactivator)	GCGATCGGCAGATCATGACTCAGGGTGACGGGTTCA
CREB responsive (general transactivator)	GCGATCGGCAGATCABNBVNTGACGTCAGGGTGACGGGTTCA
NFY responsive (general transactivator)	GCGATCGGCAGATCANNCCAATNNGGGTGACGGGTTCA
Spacer forward	GCGATCGGCAGATCANNNNNNNNNNGGGTGACGGGTTCA
Spacer reverse	TGATCTGCCGATCGCNNNNNNNNNNTGAACCCGTCACCC
AHR responsive (forward)	GCGATCGGCAGATCACCYCNRRSTNGCGTGASAGGGTGACGGGTTCA
AHR responsive (reverse)	TGATCTGCCGATCGCASAGTGCGNTSRRNCYCCTGAACCCGTCACCC
Estrogen Receptor responsive (forward v 1)	GCGATCGGCAGATCAGGTCACTGTGACCGGGTGACGGGTTCA
Estrogen Receptor responsive (forward v 2)	GCGATCGGCAGATCARGNCANNNTGACCYGGGTGACGGGTTCA
Estrogen Receptor responsive (reverse)	TGATCTGCCGATCGCCCAGTGTCACTGGTGAACCCGTCACCC
HIF responsive (forward v 1)	GCGATCGGCAGATCATCTGTACGTGACCACAGGGTGACGGGTTCA
HIF responsive (forward v 2)	GCGATCGGCAGATCAGTCTACGTGCGGACGGGTGACGGGTTCA
NF-kappaB responsive (forward v 1)	GCGATCGGCAGATCAGGGGACTTTGCCGGGTGACGGGTTCA
NF-kappaB responsive (forward v 2)	GCGATCGGCAGATCAGGGGRWYYCCCGGGTGACGGGTTCA
NF-kappaB responsive (reverse)	TGATCTGCCGATCGCCCGTTTCAGGGGTGAACCCGTCACCC
p53 responsive (forward)	GCGATCGGCAGATCAGAACATGTCCAAGCATGCTGGGGTGACGGGTTCA
PPAR-gamma responsive (reverse)	TGATCTGCCGATCGCHACTGGAAACTGGRRTGAACCCGTCACCC
PPAR-gamma responsive (forward)	GCGATCGGCAGATCARRGGTCAAAGGTCAHGGGTGACGGGTTCA
SREBP responsive (forward)	GCGATCGGCAGATCAKATCACCCCACGGGTGACGGGTTCA
SREBP responsive (reverse)	TGATCTGCCGATCGCCACCCCACTAKTGAACCCGTCACCC
p53 reverse	TGATCTGCCGATCGCGTCGTACGAACCTGTACAAGTGAACCCGTCACCC
HIF reverse	TGATCTGCCGATCGCGATCCGTCGTGCATCAGTGTGAACCCGTCACC