Cloning Promoter and intron into pGL4.23

1. PCR of the Super Core Promoter and synthetic intron

- see **attachment 1** for detailed PCR program
- backbone: any backbone containing these (human) SCP and intron (e.g. STARR-seq vector)
- primers (red are restriction enzymes kpnI and AgeI, this can be customized)

  fwd: TAACTGGCCGGTACCTGAGC
  rev: GCTACCGGTTTAAGCCTGCAGGCGACCTG

- Program
  - annealing temp: 66°C
  - extension time: 15sec
- mix:
  - phusion mix (2x) 25 µl
  - primer 1 2.5 µl
  - primer 2 2.5 µl
  - template ~1 µg
  - water to 50 µl

Note: the sequence of the SCP and intron are the written below. These are human specific. Promoter and intron can be altered to suit the organism of choice. This basic vector with SCP and intron can also be requested from our lab.

TAACTGGCCGGTACCTGagctCCCCTAGGGTACTTATATAAGGGGGTGGGGGCGCGTTCGTCCTCAGTCGCGAT
CGAACACTCGAGCCGAGCAGACGTGCCTACGGACCGGGGCCCGAATTAATTCGCTGTCTGCGAGGGCCAGCT
GTTGGGGTGAGTACTCCCTCTCAAAAGCGGGCATGACTTCTGCGCTAAGATTGTCAGTTTCCAAAAACGAGGAG
GATTTGATATTCACCTGGCCCGCGGTGATGCCTTTGAGGGTGGCCGCGTCCATCTGGTCAGAAAAGACAATCTT
TTTGTTGTCAAGCTTGAGGTGTGGCAGGCTTGAGATCTGGCCATACACTTGAGTGACAATGACATCCACTTTGCC
TTTCTCTCCACAGGTGTCCACTCCCAGGTCCAACTGCAGGTCGCCTGCAGGCTTAA

2. Purification of fragments

- see **attachment 3** for a detailed purification protocol
- gel extraction and purification
  - gel: 2 %
  - time: 30 min
  - expected lengths: 434 bp

3. Restriction of backbone and fragments

- see **attachment 1** for detailed protocol for restriction
- fragment: PCR fragment of promoter and intron
- backbone: pGL4.23
- restriction enzymes: KpnI and AgeI
- mix
  - cutsmart buffer 5 µl
  - AgeI-HF (20U) 1 µl

- KpnI-HF (20U) 1 µl
- DNA (1 µg) x µl
- Water

- Time and temp: 2h at 37°C

4. Purification of backbone and fragment

- See **attachment 3** for details on purification method
- gel extraction of backbone
  - gel: 1 %
  - time: 30 min
  - expected length: 1258 bp + 3025 bp
    - required fragment: 3025 bp
  - elution in 50 µl EB

- PCR purification
  - Promoter + intron fragment (around 416bp)
  - If needed, extra PCR purification of backbone fragment
  - Elution in 50 µl EB

5. ligation of promoter + intron into pGL4.23 backbone

- See **attachment 1** for details
- ratio vector/insert:
  - 50ng vector
  - 3x molar excess
- vector:
  - length: 3024 bp
  - concentration: 50ng
  - molarity: 25.46 fmol
- insert
  - length: 416 bp
  - molarity: 76.44 fmol
  - concentration: 20.64 ng
- mix:
  - vector 50 ng
  - insert 20.64 ng
  - buffer 10 µl
  - ligase 1 µl
  - water to 20 µl
- Time and temp:
  - 15 min at RT (at 22°C if to cold or hot in lab)
- negative control
  - only vector and no inserts

6. Transformation of ligation product

- see **attachment 2** for details
- 2-4 µl ligation product into (in-house) competent cells
- 1 µl of pUC19 as control for efficiency control
- heatshock
  - 40 sec
  - 42 °C
- incubation for 1h at 37 °C
- plating on AMPICILLIN plates
  - few different volumes plates

7. Control of product: prepping and sequencing

- see **attachment 4** for details
- Check and note the transformation efficiency
- cPCR
  - primers: RVprimer3 and EBV_rev (specific to pGL4 backbone):
    - positive: 1077 bp
    - negative: 1919 bp
  - primers RVprimer3 and fragment specific reverse primer:
    - positive: 481 bp
    - negative: 0 bp
  - mix:
    - phusion mix 5 µl
    - Primer 1 0.5 µl
    - Primer 2 0.5 µl
    - Water 4 µl
  - PCR
    - Suspend 1 colony in 10 µl LB
    - Mix 9 µl mix and 1 µl of suspended colony
    - Run at annealing temp 55°C for 25 cycles
  - Put the results on gel

- Sequencing
  - Select the correct colonies and grow overnight
  - Perform a miniprep
  - Send for sequencing with the appropriate primers

Cloning reporter into pGL4.23

1. PCR of Venus

- See **attachment 1** for details
- backbone: any backbone containing the reporter of your choice
- primers: (red indicate restriction enzymes AgeI and XbaI)

  fwd: ACTACCGGTCAGAAAAAATGGTGAGCAAGG
  Rev: GCTTCTAGATAACTTGTACAGCTCGTCCATG

- Program
  - annealing temp: 58°C
  - extension time: 30 sec
- mix:
  - phusion mix (2x) 25 µl
  - primer 1 2.5 µl
  - primer 2 2.5 µl
  - template ~1 µg
  - water to 50 µl

note: the sequence of the venus reporter is shown below. This can be altered into any reporter of choice.

TCAGAAAAAATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGC
GACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTG
AAGCTGATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGGGCTACGGCCTG
CAGTGCTTCGCCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACG
TCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGA
CACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTG
GAGTACAACTACAACAGCCACAACGTCTATATCACCGCCGACAAGCAGAAGAACGGCATCAAGGCCAACTTCAA
GATCCGCCACAACATCGAGGACGGCGGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGA
CGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCTACCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAG
CGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGT
TA

2. Purification of fragments

- see **attachment 3** for details on the method
- gel extraction and purification
  - gel: 1.2 %
  - time: 30 min
  - expected lengths: 746 bp

3. Restriction of backbone and fragments

- see **attachment 1**
- fragment: PCR fragment of venus
- backbone: pGL4.23 with promoter and intron
- restriction enzymes: AgeI and XbaI
- mix
  - cutsmart buffer 5 µl
  - AgeI-HF (10U) 0.5 µl

- KpnI-HF (10U) 0.5 µl
- DNA (1 µg) x µl
- Water to 50 µl

- Time and temp: 2h at 37°C

4. Purification of backbone and fragment

- See **attachment 3**
- gel extraction of backbone
  - gel: 1 %
  - time: 30 min
  - expected length: 526 bp + 2915 bp
    - required fragment: 2915 bp
  - elution in 50 µl EB

- PCR purification
  - Venus fragment (around 746 bp)
  - Elution in 50 µl EB

5. Ligation of venus into altered pGL4.23 backbone

- See **attachment 1**
- ratio vector/insert:
  - 50ng vector
  - 3x molar excess
- vector:
  - length: 2915 bp
  - concentration: 50ng
  - molarity: 26.43 fmol
- insert
  - length: 746 bp
  - molarity: 79.29 fmol
  - concentration: 38.39 ng
- mix:
  - vector 50 ng
  - insert 38.39 ng
  - buffer 10 µl
  - ligase 1 µl
  - water to 20 µl
- Time and temp:
  - 15 min at RT (at 22°C if to cold or hot in lab)
- negative control
  - only vector and no inserts

6. Transformation of ligation product

- See **attachment 2**
- 2-4 µl ligation product into in-house competent cells
- 1 µl of pUC19 as control for efficiency
- Heatshock
  - 40 sec
  - 42 °C
- Incubation for 1h at 37 °C
- Plating on AMPICILLIN plates

7. Control of product: prepping and sequencing

- See **attachment 4**
- Check and note the transformation efficiency
- cPCR
  - primers RVprimer3 and EBV_rev:
    - positive: 1292 bp
    - negative: 1077 bp
  - mix:
    - goTaq buffer 2 µl
    - Primer 1 0.5 µl
    - Primer 2 0.5 µl
    - goTaq 0.1 µl
    - dNTPs 0.4 µl
    - Water 6.5 µl
  - PCR
    - Suspend 1 colony in 10 µl LB
    - Mix 9 µl mix and 1 µl of suspended colony
    - Run at annealing temp 55°C for 25 cycles
  - Put the results on gel

- Sequencing
  - Select the correct colonies and grow overnight
  - Perform a miniprep
  - Send for sequencing with the appropriate primers

Generating a barcode pool

1. Enzymatic inverse PCR to clone in the barcode

- See **attachment 1**
- backbone: pGL4.23+SCP+Intron+Venus
- primers: (red indicates the AscI restriction site)

  fwd:
  GTCGGCGCGCCGATCNNNNNNNNNNNNNNNNNGCTTCGAGCAG
  ACATGATAAGATAC
  Rev: TATGGCGCGCCTTACTTGTACAGCTCGTCCATGC

- Program
  - annealing temp: 56°C
  - extension time: 2.5 min
  - cycles: 35
- mix:
  - phusion mix (2x) 100 µl
  - primer 1 10 µl
  - primer 2 10 µl
  - template 50 ng
  - water to 200 µl

- prep the mix and divide over 4 eppies.

2. Purification steps

- see **attachment 3**
- PCR purification
  - Each PCR reaction on 1 column (4 columns)
  - Double elution with 30 µl EB
  - Aim at 10-15 µg total yield (in around 100-120 µl)

3. Restriction

- See **attachment 1**
- fragment: inverse-PCR fragment of backbone with synIntron + SCP
- restriction enzymes: AscI (NEB)
- total of 16 reactions, pooled per 2 reactions (divide the total volume of DNA over all 16 reactions, aiming at around 0.8-1 µg per reaction maximum)
- mix for 1 reaction
  - cutsmart buffer 5 µl
  - AscI (20U) 0.5 µl
  - DNA (1 µg) x µl
  - Water to 50 µl
- Time and temp : 2h at 37°C
- 15 min before end add 1 µl DpnI per 200 µl reaction.

4. Purification of backbone and fragment

- see **attachment 3**
- PCR purification
  - Each restriction reaction on 1 column (8 columns)
  - Double elution with 30 µl EB
  - Aim at around 60% of started input DNA as total yield (in around 200-240 µl)

5. Ligation of linearized and restricted barcoded backbone

- See **attachment 1**
- vector:
  - length: 3670 bp
  - dilute vector to 2 ng/µl reaction. Eg 8 µg vector will be ligated in a total volume of 4 ml
- 1 µl ligase is used per 100 ng vector
- Adjust number of total reactions to total volume needed for 2 ng/µl dilution.
- mix (1 reaction):
  - vector 1 µg
  - buffer 50 µl
  - ligase 10 µl
  - water to 500 µl
- Time and temp:
  - Overnight at 16 °C

6. Purification of ligated barcoded pool

- See **attachment 3**

A. Speedvac volume reduction

- Each ligation reaction (500 µl) needs to be reduced in volume by speedvac (9th floor O&N1)
  - 2h of speedvac will reduce 500 µl to 100 µl

B. Option 1: double PCR purification

PCR purification 1

- each ligation reaction is purified over 1 column
- Elution with 30 µl EB. Each 30 µl is used for 2 columns.
  - Eg 8 columns = 4x 30 µl EB
  - Should try to get over 90% recovery. Possibly still some ethanol contaminations

PCR purification 2

- One elution over two columns
- Double elution with 30 µl EB per column
  - Aim at around 30% recovery of total ligated DNA

- 260/230 should be at a good level to ensure as little ethanol contamination as possible.

C. Option 2: ethanol precipitation and PCR purification

Ethanol precipitation

- mix (per 300 µl fractions)
  - 2.4 µl glycogen
  - 30 µl Sodium Acetate (3M)
  - 900 µl 100 % ethanol (chilled)
- follow protocol (attachment 3)
  - put at -80°C for 2h instead of overnight -20°C
- dissolve the pellet with 200 µl water or EB per fraction

PCR purification

- Each fraction of precipitated DNA over 1 column
- Double elution in 30 µl EB per fraction
  - Aim at around 70% recovery of the initial ligated vector (in a total volume of around 100 µl)

Note: option 2 has yielded the most pure DNA

7. Electroporation of the barcoded backbone

- See **attachment 2**
- Electroporation
  - Around 100-120 ng per 20 µl electro-competent cells
    - Aim at electroporating around 1.5-2 µg DNA
  - Add 1 positive control with 1 µl pUC19
  - Follow the electroporation protocol and its settings (attachment 2)
    - Aim at time constants higher then 4 (indicates pure DNA and thus an efficient electroporation)
  - Pool all the recovered electroporations and grow overnight at 37°C in 0.5 liter per 3 electroporations
  - Plate dilutions
- Collection of pellet
  - Collect 3-4 electroporations per 50 ml tube and aim at a weighted pellet of around 2-3 gr per tube.
    - First use the big centrifuge (200 ml tubes) and rinse the tubes and collect the pellets in 50 ml tubes
- Transformation efficiency
  - Aim at an efficiency of 0.5 – 1 million CFUs per ng DNA
  - Aim at electroporating 1.5 to 2 µg DNA
  - Final aim should be at least 5% of total variability
    - Total variability is 4^17 = 1.7 x10^10 CFUs

8. Control of product

- See **attachment 4**

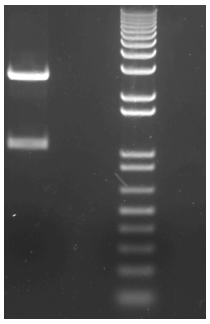
Miniprep

- Collect the colonies from the 1/10 dilution plate and prep

- If wanted/needed collect a few single colonies

Restriction

- backbone: barcoded backbone pool
- neg control: backbone pGL4.23 + SCP + Intron + Venus
- restriction enzymes: Kpn-HF and AscI-HF
- mix
  - cutsmart buffer 2 µl
  - AscI (10U) 0.5 µl
  - KpnI-HF (10U) 0.5 µl
  - DNA (1 µg) 1 µl
  - Water to 10 µl
- Time and temp : 2h at 37°C
- Expected
  - Neg control: 3649 bp
  - Barcoded pool: 2389 bp + 1150 bp



sequencing

- minipreps of barcoded backbone pool
- primers: design as wanted

9. Extracting DNA of barcoded backbone pool

- Giga prep
  - Aim at 200-400 ng/µl (with a total of 1-2 mg DNA)
  - Total volume of around 5 ml

Preparing the input for infusion

1. Retrieving the input

sheared DNA fragments

- Purify genomic DNA
- Shear the DNA using the covaris aiming at 500-1000 bp length

Note: other methods to retrieve input are also possible (e.g. ChIP)

2. Library preparation to add in-fusion compatible adapters

- See **attachment 5** for details on the method
- end-repair
  - between 250 ng and 5 µg of input DNA in reaction volume of 100 µl
  - elution in 25+20 µl EB
- dA-Tailing
  - reaction in 50 µl
  - PCR purification and elution into 25 µl
- adapter ligation
  - prepare fresh adapters and dilute to 15 µM
  - adapter sequences:
    - CCATCTCATCCCTGCGTGTCTCCGACTCAG*T
    - CTGAGTCGGACACGCAACAGGGGATAG
  - total volume of 50 µl
  - double purification with beads
    - 1st round 1:1 ratio
    - 2nd round: 0.8:1 ratio
  - elution with 50 µl EB

3. PRC amplification

- see **attachment 1**
- input: fragmented and adapter ligated gDNA
- primers: (red indicates the primer tails)

  fwd: ATCTGTGTGTTGGTTCCATCTCATCCCTGCGTGTC
  Rev: GTACCGGCCAGTTAGCTATCCCCTGTTGCGTGTC

- Program
  - annealing temp: 60°C
  - extension time: 30 sec
  - final extension: 1 min
  - cycles: **10-15** (18x for ChIP-fragments)
- mix:
  - Kapa HIFI mix (2x) 25 µl
  - primer 1 2.5 µl
  - primer 2 2.5 µl

  - template up to 50 ng
  - water to 50 µl
- perform the PCR once to check the number of cycles
- repeat the PCR with the correct number of cycles in 5-fold (250 ng input total)
- if input is ChIP-fragments skip the purification steps and go straight to step 6. Bead purification

4. Bead purification

- see **attachment 3**
- 1st round 0.7 bead:DNA ratio
- 2nd round 0.6 bead:DNA ratio
- Final elution in 20 µl EB

5. DNA enrichment protocol

- Design regions of interest to capture and enrich for from gDNA
- Follow the protocol of the company used for DNA capture (we used Mybaits)
- The input DNA should be between 100-500 ng in 6 µl
  - Use the vacuum centrifuge if needed
- For the final amplification step use primers

  - fwd: ATCTGTGTGTTGGTTCCATCTCATCCCTGCGTGTC
  - Rev: GTACCGGCCAGTTAGCTATCCCCTGTTGCGTGTC

6. Purification

- see **attachment 3**
- beads
  - 1st round 0.7 bead:DNA ratio
  - 2nd round 0.6 bead:DNA ratio
  - Final elution in 50 µl EB
- PCR
  - Elution in 50 µl
- Aim at 60-100 ng/µl

7. Pooling

- pool enough individual captures until at least 250 ng is collected
- if possible do a small qPCR to check fragment enrichment

Preparing the barcoded library pool for infusion

1. Enzymatic inverse PCR to linearize backbone

- see **attachment 1**
- backbone: pGL4.23+SCP+Intron+Venus with barcode pool
- primers: (red indicates the compatible 15 bp ends for infusion)

  fwd: CTAACTGGCCGGTACCTGAG
  Rev: AACCAACACACAGATGTAATGAAAA

- Program
  - annealing temp: 63°C
  - extension time: 1 min 40 sec
  - cycles: 35
- mix:
  - phusion mix (2x) 25 µl
  - primer 1 2.5 µl
  - primer 2 2.5 µl
  - template 25-50 ng
  - water to 50 µl
- Perform 2-4 reactions simultaneously.

2. Purification steps

- See **attachment 3**
- gel extraction
  - expected length: ~3500 bp
  - two PCR reactions were extracted on one column
  - elution in 40 µl EB

3. DpnI restriction

- see **attachment 1**
- fragment: inverse-PCR fragment of barcoded backbone
- mix for 1 reaction
  - cutsmart buffer 5 µl
  - DpnI 1 µl
  - DNA 40 µl
  - Water 4 µl
- Time and temp: 1h at 37°C

4. Purification

- See **attachment 3**
- gel extraction
  - expected lengt: ~3500 bp
  - Elution in 30 µl (double)
- PCR purification
  - Two gel extractions on one column

- Elution in 50 µl
- Minelute
  - Elution in 15 µl
- Aim at 100-200 ng/µl

Generating the final enhancer-barcode library

1. Infusion reaction

A. Preparations

vector:
- weigth 100 ng
- size 3539 bp
- molarity 43.54 fmol

insert (captured DNA):
- 1:2 ratio with vector
- molarity 87.1 fmol
- size: 1 kb
- needed: 56.53 ng

B. Reaction

	1 reactions	neg	positive
insert	56.53 ng	/	2 µl
vector	100 ng	100 ng	1 µl
enzyme	2 µl	1 µl	1 µl
water	to 10 µl	to 5 µl	to 5 µl

- set at 50°C for 15 min
- let cool to 4°C

2. Precipitation of DNA

- see **attachment 3**
- dissolve in 20 µl
- aim at 20-40 ng/µl

3. Transformation

- see **attachment 2**

A. Preparations

- Preheat the following at 37°C
  - several liters of LB
  - recovery medium for after electroporation
  - plates (+Amp)
- Prechill and prepare the following:
  - electroporation cuvettes,
  - eppendorf tubes (5xlibrary (for 12.5 µl), 3x for all controls)
- Prepare the following
  - 15 ml tubes (1 per electroporation)
  - tips

- towels
- SOC medium and eppendorfs for the dilutions

B. Electroporation

- Around 100 ng per 20 µl electorcompetent cells
  - Add 1 positive control with 1 µl pUC19
- Follow the electroporation protocol and its settings (attachment 2)
  - Aim at time constants higher then 4 (indicates pure DNA and thus an efficient electroporation)
- Pool all the recovered electroporations and grow overnight at 37°C in 0.5 liter per 3 electroporations
- Plate dilutions (see attachment 4)

4. Harvesting

- Collect 3-4 electroporations per 50 ml tube and aim at a weighted pellet of around 2-3 gr per tube.
  - First use the big centrifuge (200 ml tubes) and rinse the tubes and collect the pellets in 50 ml tubes
- Transformation efficiency
  - Aim at an efficiency of > 1 million CFUs

5. Control

- See **attachment 4**

Miniprep and sequencing

- Collect around 20-50 single colonies

qPCR for expected fragments

6. Final library

If controls are ok then prep all the collected pellet using a giga prep.

Transfection of Cheq-Seq libraries

- See **attachment 6** for general details of the method

1. Plating cells

- Plate 5 million cells per 15 cm plate (confluency of 70%-80%)
- Provide enough plates for perturbation and control
    - Include a plate for a GFP control vector as a TFX control

2. Transfection

- transfect 37 µg of DNA per plate
- reagent: a 1:1.2 ratio of DNA:PEI (37 µg -> 45 µl)
- prep each in a total volume of 1500 µl per plate
- combine both mixes into a 3ml transfection volume per plate.
- Include a GFP control

3. perturbation

- If required perform the proper perturbation of your TF of interest

4. Collection of cells

- Depending on the perturbation, collect your cells x hours after transfection
- Wash the cells with PBS
- trypsinize the cells and count the number of cells
- split the plate and take 1/6 for plasmid extraction and the rest for RNA extraction

Checking transfection

1. plasmid extraction

- see **attachment 7**
- use 1/6 of the plate to extract plasmid from using the plasmid plus extraction kit (qiagen)
- don't expect more then 2% of input DNA

2. qPCR check

- see **attachment 4**
- compare the extracted plasmid DNA after and before transfection
- use venus as a total plasmid control
- evaluate a few regions before and after transfection

Generating the quantitative libraries of the barcode

1. RNA extraction

- see **attachment 7**
- Extract around 5/6 transfected plate.
- Follow the normal RNA extraction protocol
  - Expect around 10 µg per 1 million cells
- Continue with an mRNA extraction to select on translated RNA
  - Expect to retrieve around 4% of the input RNA
  - Immediately continue to cDNA

2. cDNA preparation

- See **attachment 7**
- follow the cDNA preparation protocol
  - don't do more then 1 µg per reaction
- after reaction purify with qiagen minelute columns
  - expect around 5% of the input mRNA as output

3. PCR for generating the barcode fragments for sequencing

A. first round PCR

- see **attachment 1**
- backbone: cDNA of transfected Cheq-Seq library
- primers: (red are variable length barcodes if multiplexing is needed)
  - N between 0 and 7 bp

fwd: CCTACACGACGCTCTTCCGATCT(X)$_N$AGCTGTACAAGTAAGGCG
rev: CAGACGTGTGCTCTTCCGATCTTGTATCTTATCATGTCTGCTCGAA

- Program
  - annealing temp: 56°C
  - extension time: 8 sec
  - final extension: 5 minutes
  - 10 cycles
- mix:
  - phusion mix (2x) 25 µl
  - primer 1 2.5 µl
  - primer 2 2.5 µl
  - template up to 100 ng per reaction
  - water to 50 µl

- column PCR purification with elution in 30 µl (2x15 µl)

B. second round PCR

- see **attachment 1**

- backbone: product from primer round one
- primers: (red is an indexing barcodes, these are illumine primers)

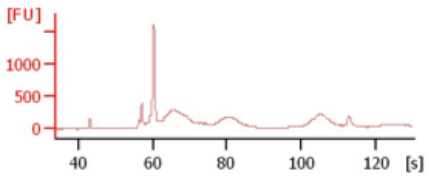
FWD AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC*T

REV CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCxT

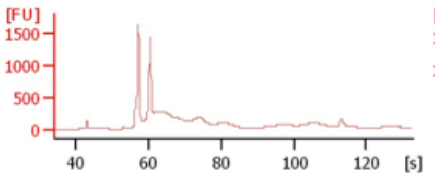
- Program
  - annealing temp: 64°C
  - extension time: 10 sec
  - final extension: 5 minutes
  - 10 cycles
- mix:
  - phusion mix (2x) 25 µl
  - primer 1 2.5 µl
  - primer 2 2.5 µl
  - template up to 100 ng per reaction
  - water to 50 µl

4. Purification of library

- see **attachment 3**
- beads
  - 1st round 1:1 bead:DNA ratio
  - control on bioanalyzer
    - expected length is 190 bp
  - 2nd round if dimers (0.9:1)
  - Final elution in 25 µl EB



good



bad (dimers)

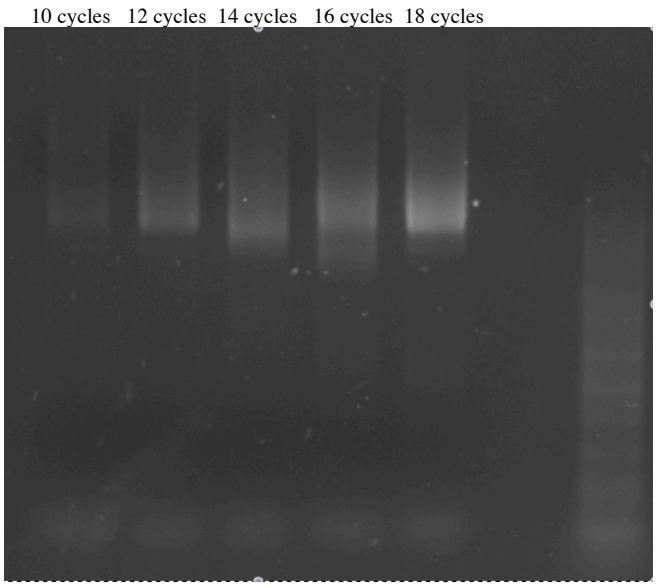
Generating the enhancer-barcode libraries

1. test PCR for linearizing the input library

- see **attachment 1**
- backbone: library pool used as input for TFX
- primers:
- to do a test to determine cycles
  - take 10 µl aliquot at 10, 12, 14, 16 and 18 cycles

fwd: CCTACACGACGCTCTTCCGATCTTGTGTTGGTTCCATCTCATCC
rev: CAGACGTGTGCTCTTCCGATCTTCATCAATGTATCTTATCATGTCTGC

- Program
  - annealing temp: 65°C
  - extension time: 1 min 5 sec
  - final extension: 5 minutes
  - up tot 18 or 20 cycles
- mix:
  - phusion mix (2x) 25 µl
  - primer 1 2.5 µl
  - primer 2 2.5 µl
  - template up to 100 ng per reaction
  - water to 50 µl
- expected results on gel are between 2-3 kb
  (in example 14 cycles is optimal)



2. PCR for linearizing the input library

- see **attachment 1**
- backbone: library pool used as input for TFX

- primers:

  fwd: CCTACACGACGCTCTTCCGATCTTGTGTTGGTTCCATCTCATCC
  rev: CAGACGTGTGCTCTTCCGATCTTCATCAATGTATCTTATCATGTCTGC

- Program
  - annealing temp: 65°C
  - extension time: 1 min 5 sec
  - final extension: 5 minutes
  - optimal number of cycles determined in step 1
- mix:
  - phusion mix (2x) 200 µl
  - primer 1 20 µl
  - primer 2 20 µl
  - template up to 1 µg per 8 reactions
  - water to 400 µl

4. Purification of library

- see **attachment 3**
- beads
  - 1st round 1:1 bead:DNA ratio
  - control on bioanalyzer/gel
    - expected length is 2-3 kb
  - 2nd round if dimers
  - Final elution in 40 µl EB

- should aim at 1-2µg of final DNA to send to the company or genomics core to perform PacBio library prep and sequencing.

Attachments

Attachment 1

PCR

- Make your PCR mix. Add water last in order to mix well.

	All-in mix	**Basic mix**
buffer	25 µl	10 µl
Primer 1	2.5 µl	2.5 µl
Primer 2	2.5 µl	2.5 µl
dNTPs	/	2 µl
polymerase	/	1 µl
template	X µl	X µl
Water	To 50 µl	To 50 µl

- Use the following program on the thermocycler. Adjust annealing temperature and extension time according to primers, fragment length and the polymerase used.

	temp	**time**	
Initial denaturation	98°C	30 sec	
denaturation	98°C	10 sec	35x
Annealing	55°C	15 sec	
Extension	72°C	2 min	
Final extension	72°C	5 min	
hold	4°C	~	

Restriction

- Make the following mix

Buffer (10x)	2.5 µl
Restriction enzyme	5 units
BSA (optional)	0.25 µl
template	Up to 0.5 µg
water	To 25 µl

- Mix well by pipetting up and down
- Incubate at 37°C for 1-2h (or according to specifications)
- Terminate the reaction
    - By putting on gel
    - By continuing on to a PCR purification

- ***Notes***
    - Maximum 1 µg DNA per 50 µl total volume
    - 5-10 units to cut 1 µg DNA
    - Take care of star activity when adding a lot of enzyme (> 10%)

- With a double digest avoid buffers where one of the enzymes had less then 50% activity.

Ligation

- Make the following mix. Take care to add the vector to insert in a 1:3 ratio.

Buffer (10x)	2 µl
vector	X ng
insert	Y ng
ligase	1 µl
water	Up to 20 µl

- Mix by pipetting up and down
- Put at 16°C overnight or RT for 10min to 2h
- Heat inactivate at 65°C for 10 min
- Put on ice or freeze at -20°C until ready to continue

- Note: for calculating 1:3 molarity you can use for instance http://www.sciencelauncher.com/mwcalc.html

Attachment 2

Electroporation

***Note**: this is best done with two people when transforming a lot of DNA*

A. preparation on bench

- Divide your input per 2.5 µl (max 100 ng) in prechilled eppies.
    - do the same for the positive and negative control.
    - add 1 µl of pUC19 as a transformation control (10pg/µl)
- thaw the bacteria (MegaX DH10BTM T1R ElectrocompTM Cells)
- pipet 20 µl of bacteria to each tube with library (don't pipet up and down)
- transfer the bacteria + DNA to the cuvettes.
    - be quick and tap each cuvette so that the mix sinks to the bottom.

B. Electroporation

- Go to the electroporator
    - make sure it is yours and set it correctly in advance
    - conditions: 2.0kV, 25µF and 200 ohm
- Wipe each cuvette carefully so that it is dry.
    - water will make it pop and explode, losing your samples!!
- Put it in the electroporator and press the two buttons together until you hear the buzz-sound
- Take out the vial and add 1ml of recovery medium
    - ***get someone to help you here: one to electroporate, one to add the medium!***
    - *don't do more then 10 electroporations on your own*
- Only pipet once up and down and transfer to 15 ml tube
- Put at 37°C for 1h
- After 1h plate or transfer to a bigger culture to grow bulk overnight.

Heat-shocking

- Preheat LB
- Thaw chemically competent cells on ice
- Add DNA to the cells
    - Up to 100 ng, in max 5 µl per 50 µl cells
- Incubate for 30 min on ice
- Heat shock at 42°C for 30-45 sec

- Incubate on ice for 2 min
- Add 950 µl preheated LB
- Incubate shaking at 37°C for 1h
- After 1h plate or transfer to a bigger culture to grow bulk overnight.

Plating and growing the transformed bacteria

- Put a few tubes with LB medium at 37°C in advance
- Prepare dilutions as a control for transformation efficiency (optional).
    - prepare the dilutions using SOC medium

	library	negative	positive	pUC19
1/10	900 µl	900 µl	900 µl	900 µl
1/50	400 µl	400 µl	400 µl	400 µl
1/500	900 µl	900 µl	900 µl	900 µl
1/5000	900 µl	900 µl	900 µl	900 µl

        - add 100 µl of the library to the first eppendorf (1/10 dilution)
        - pipet up and down, vortex and pipet up and down a few more times
        - put 100 µl into the second eppendorf (1/50 dilution), etc …
        - take 100 µl of each and spread it on a plate
        - put all the plates at 37°C ON

- after taking 100 µl for dilution pool all of the library together if applicable.
- add your transformation to the preheated LB
- Put overnight at 180 rpm at 4°C (about 12-13 hours)
- Alternatively just plate all your transformed bacteria on preheated plates

Note: formula to calculate efficiency

$$\frac{\#\text{ CFU}}{\text{conc in pg}} \times 10^6 \times \frac{\text{total volume of transformation}}{\text{volume plated}} \times \text{dilution factor}$$

total volume: total transformation volume eg is you do 4 transformation then you pool 4x 1ml and take 100 µl from that to plate
dilution factor: eg 10^2 if dilution was 1/100

Attachment 3

PCR and minElute purification

- Add a 5x excess amount of PB buffer
- Mix well by pipetting then load the sample on the column
- Wash with PE.
    - Optional: After centrifuge one extra time for 2 minutes full speed to remove residual ethanol
- Elute using desired amount of EB. Pipet this right on the membrane (up to 50 µl).
- If needed, repeat this elution step using the eluant from the first elution.

Gel extraction and purification

A. running a gel

1.2% agarose gel > 600 bp
2% agarose gel < 600 bp

- weight 1gr of agarose for 100 ml TAE(1x) buffer
- dissolve in buffer while heating in the microwave
- regularly swirl while heating
- when fully dissolved, cool the mixture a bit under the tap until 'touchable'
- add 1/10000 (10 µl/100ml) of sybr safe
- pore in the gel holder and put the comb in the gel
- let set for ~30 minutes
- add loading dye to your samples (1/6)
- load your samples in the wells (max 20-25µl)
- load a ladder if needed
- run the gel for around 20-30 min
- look under the blue light for the right signal
-

note: when extracting DNA from a gel avoid visualizing under UV

B. extracting from a gel

- use a clean scalpel blade to cut out the needed fragment
- put in a clean eppie
- weight the gel
- add 3x the gel weight of buffer QG (eg 100 mg → 300 ml QG)
- incubate at 50°C for 10 minutes
    - alternatively spin the gel on a rotar at RT until dissolved
    - if color is not yellow, add 5-10 µl 3M sodium acetate
- add 1 gel weight of 100% isopropanol
- mix well and put on the column
    - optional, add another 500 µl of QG as a wash step

- wash with 750 µl PE and spin for 1 min at 12000 rpm
- spin dry for another 1-2 min
- put column in a clean eppie and add up to 50 µl EB on the membrane
    - let it incubate for 1 minute and spin down.
    - If needed, repeat the elution with the eluent.

Ethanol precipitation

Work ON ICE!!

- pool all four samples and top it off to 250 µl with EB (~ 210 µl).
    - do the same for control sample, if applicable
- add 25 µl of 3M sodiumacetate (pH 5.2)
- add 2 µl of glycogen. It will colour your pellet white
- add 750 µl 100 % EtOH (stored at -20°C)
- vortex the samples
- precipitate ON at -20°C (or at least 7-8h)
    - alternatively put at -80°C for 2h
- vortex the samples
- centrifuge at max. speed for 15 min. DO NOT discard supernatans
- vortex the tubes. The pellets should float in the fluid
- centrifuge and discard supernatans (a small amount can be left)
    - this double centrifugation is needed to collect all DNA
- wash 1x with 1ml 70% EtOH (ice cold, stored at -20°C)
    - don't pipet up and down. once the pellet touches the tip it will stick to it
- vortex the samples and centrifuge at max. speed for 15 min at 4°C.
- Remove the supernatans.
- air dry the pellet (approx. 3min).
    - the pellet should become transparent
    - don’t wait too long because then the resuspension will be very hard.

- Dissolve the sample in water or EB
    - pipet a few times but don't actually take the pellet in your tip (shearing).
- Vortex the samples for about 2 sec (lower speed) and spin down
- Put on 37°C for 15 seconds. Then put immediately on ice for 1 min
- Vortex the samples for 2 sec on slow speed (hold them close to bottom) and spin down.
- Transfer to the -80°C for 3-4 h immediately
- After you can store at -20°C (at least a week)

Bead purification

--> beads at RT 30 minutes in advance
--> magnet is rinsed with EtOH

- Pool reactions if needed
- Combine the beads with the sample by pipetting up and down a few times
- Votrex the sample (approx. 10 sec). Leave on RT for 15 '
- Put on magnet for 10 min. After 8 min swing the magnet with sample really hard from left to right to ensure all the sample in the lid is in the tube.
- Pipet the supernatans slowly and discard.
- Wash with 80% EtOH by pipetting it against the opposite eppie-wall to not disturb the pellet.
- Repeat the wash step 2x
- Let the beads dry for a bit (5-10 minutes) just until the very glossy-ness is gone
- Add EB in a 10 µl/reaction ratio. Eg if about 5 samples were pooled this means a 50 µl elution in EB.
- Resuspend the beads and vortex. Put on 37°C, shaking for 3 '
- Put in the magnet by slightly tilting the magnet and then sliding the eppie in the slot whilst under this tilted angle.
- Leave on RT for 1 min
- Pipet and KEEP the supernatans and put it in a clean eppie

Attachment 4

cPCR

A. prepare bacterial culture:

- add 10 µl LB to a PCR-tube.
- Put a tip (1-10µl) on you pipet and briefly tap your colony
- Go with this tip in the LB.
- Pipet up and down and rub the side of the tube a little bit to make sure the bacteria are spread in the medium

B. prepare the PCR reaction

- Make the PCR mix

for 1 colony PCR reaction (on ice):

1x buffer (5x) (green)	2µl	
0.2mM dNTPs	0.4µl	
0.5µM P1		0.5µl
0.5µM P2		0.5µl
goTaq polymerase		0.1µl
water		6.5 µl
		10µl

- Distribute 10 µl in new PCR tubes
    - *Note: standard buffer that I use is the gotaq GREEN buffer because then I don't have to add loading dye to put it on gel later.*
- take 1 µl from the bacteria +LB and transfer it to the PCR mix.
- put on the following program:

Initial denaturation	95°C	2 min	
Denaturation	95°C	45sec	]
Annealing	55°C*	45sec x	] 30
Extension	72°C	Ymin*	]
Final extension	72°C	5min	
Hold	4°C	99min	

* anealing temperature is dependent on the melting temperature of your primers. Select the lowest melting temperature of the two primers as annealing temp. (in case of M13fwd –rev, we do 55°C)
*the extension time depends on the polymerase used. In general goTaq uses about 1min/kb. So for a fragment around 300bp I would select 30sec.

note: You can take along a positive control to check whether colony PCR worked.

C. reading and collecting the results of the cPCR

read out the results of the cPCR on a gel

If colony PCR is succesfull you can do two things.

A. add the remaining 9µl of bacteria +LB to a larger culture with needed anitbiotics (eg. 3ml) and grow overnight
B. plate the remaining 9µl of bacteria + LB on a plate (with antibiotics) and let grow overnight.

***Note**: It is important that the bacteria do not stay in the small amount of LB overnight because it will dry out and you will loose the bacteria*

Sequencing

Follow the instructions of your sequencing core or company

Attachment 5

Library prep

Starting Material: 250ng –5 µg of fragmented DNA with length of 500 bp

A. End Repair of Fragmented DNA

1. **Mix the following components in a sterile microfuge tube:**

Fragmented DNA	1–85 µl
NEB Buffer (10X)	10 µl (1x)
dNTPs (1 mM)	2 µl (20 µM)
PNK enzyme (10 000 U/ml)	5 µl (50 U)
T4 DNA polymerase (3000 U/ml)	5 µl (15 U)
Klenow	1 µl
Sterile H_2O	fill to 100 µl
---	---
Total volume	100 µl

2. **Incubate in a thermal cycler for 30 minutes at 20°C.**

3. **Purify DNA sample using qiagen PCR purification kit**

   → elute with EB in a total of 45 µl:
   elution 1: 25 µl
   elution 2: 20 µl

B. dA-Tailing of End Repaired DNA

1. **Mix the following components** in a sterile microfuge tube:

End Repaired, Blunt DNA	42 µl
NEBNext dA-Tailing Reaction Buffer (10X)	5 µl
Klenow Fragment (3´→ 5´ exo–)	3 µl

Total volume	50 µl

2. **Incubate in a thermal cycler for 30 minutes at 37°C.**

3. **Purify DNA sample** using qiagen PCR purification kit with **Minelute** columns

   → elute with EB in a total of 25 µl

C. Adaptor Ligation of dA-Tailed DNA

1. **Mix the following components in a sterile microfuge tube:**

dA-Tailed DNA		25 µl
Quick Ligation Reaction Buffer (5X)	10 µl	
NEBNext Adaptor*	10 µl	
Quick T4 DNA Ligase		5 µl
Total volume		50 µl

use self annealed adapters. Mix 5 µl of each primer and add 0.4 µl of 5M NaCl and run the annealing program.

Adapters:

FWD	CCATCTCATCCCTGCGTGTCTCCGACTCAG*T
REV	CTGAGTCGGACACGCAACAGGGGATAG

2. **Incubate in a thermal cycler for 15 minutes at 20°C.**

D. Clean Up Using AMPure XP Beads

1. Vortex AMPure XP beads to resuspend.
2. Bead to sample ratio should be about 1.0 Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Put the tube/pcr plate on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.
5. Add 200 µl of 80% freshly prepared ethanol to the tube/pcr plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
6. Repeat Step 5 once.
7. Air dry beads for 10 minutes while the tube/PCR plate is on the magnetic stand with the lid open.
8. Elute DNA target by adding 50 µl EB to the beads. Mix well on a vortex mixer or by pipetting up and down, and put the tube/pcr plate in the magnetic stand until the solution is clear.
9. Transfer supernatant to a new tube/well

10. Repeat the protocol for a bead / DNA ration of 0.8. elute second time in 20 µl

Attachment 6

Preparation of cells

- Thaw cells enough in advance
- Expand them until enough to perform experiment (at least 15 million (3x15 cm))

Plating cells

- Wash and trypsinize the cells
- Collect them and count the cells
- Plate between 5-10 million cells in a 15 cm plate (aim at 75-80% confluency)
    - Provide 1 plate for checking transfection efficiency (plasmid extraction)
    - For RNA extraction: 1 plate for control and 1 for perturbation
    - Optional: non transfected plate and a plate for TFX control

Transfection

- 24 hours after plating cells
- combine the DNA and the PEI in seperate eppies (per plate)

DNA	optimem		PEI	optimem
37 µg	Up to 1500 µl		45 µl	1458 µl

- Combine the PEI and DNA together and let it stand for 15 minutes
- Pipet carefully and add 3 ml to a 15 cm plate

Perturbation

- Perform a perturbation as needed

Attachment 7

Plasmid extraction

Follow the Qiagen plasmid mini prep kit

- Resuspend cells in 250 µl buffer P1
    - 1x 15 cm plate
- Add 250 µl buffer P2 and invert the tube 4-6x until fully mixed
- After 4 minutes, add 350 µl buffer N3 and mix
- Centrifuge for 10 min at 13000 rpm
- Add the supernatants to the spin column
- Centrifuge at max speed for 1 minute
- Wash the spin column with 500 µl PB buffer and centrifuge for 1 min at max speed
- Wash the column with 750 µl PE buffer and centrifuge 1 min at max speed
- Spin for an additional 2 minutes to remove residual buffer
- Place the spin column in a new eppie and elute with 50 µl EB buffer/water. Let it stand for 1 minute and centrifuge for 1 minute max speed.

RNA extraction

follow the protocol of the manufacturer

Qiagen

- collect cells by trypsinization and pellet
    - no more then 10 million cells per column
- add RLT buffer
    - 1 ml RLT + 10 µl ß-mercapto
    - < 5 million 350 µl
    - > 5 million 600 µl
- add the lysate into the shredder column and centrifuge for 2 min at max speed
- add 1 volume of 70% ethanol to the lysate, mix well and transfer to the spin column
- centrifuge for 30 sec at 12000 rpm. Discard the flow through
- add 700 µl RW1 buffer and centrifuge for 30 sec at 12000 rpm
- add 500 µl RPE buffer and centrifuge for 30 sec at 12000 rpm
- centrifuge the column an additional 2 minutes at 12000 rpm
- put the column in a clean eppie and add 30-50 µl water or EB buffer to the center of the column membrane.
- Centrifuge for 1 min at 10000 rpm

Analytic jena

- Collect cells by trypsinization and pellet
    - No more then 5 million cells per column
- Add 400 µl RL to the pellet and incubate for 5 minutes, resuspending occasionally
- Add lysate to spin filter D and centrifuge for 2 min at 12000 rpm

- add 1 volume of 70% ethanol to the lysate, mix well and transfer to column R
- centrifuge for 2 min at 12000 rpm. Discard the flow through
- add 500 µl HS buffer and centrifuge for 1 min at 12000 rpm
- add 750 µl LS buffer and centrifuge for 1 min at 12000 rpm
- centrifuge the column an additional 3 minutes at 12000 rpm
- put the column in a clean eppie and add 30-80 µl water to the center of the column membrane. Incubate for 1 min at RT
- Centrifuge for 1 min at 8000 rpm

mRNA extraction

washing beads

- resuspend the beads to homogenize the mixture and transfer up to 1 mg beads (200 µl) to an eppie
- put the beads on a magnet and wait for them to move to the side.
- Remove the supernatant and remove the eppie from the magnet stand
- Resuspend the beads in 100 µl binding buffer and put back on the magnetic stand for 1-2 min
- Remove the supernatant and resuspend the beads in 100 µl binding buffer

Dynabeads mRNA extraction

- Dilute your sample to 75 µg/100 µl
- Add 100 µl of binding buffer and heat for 5 minutes at 65°C. place on ice
    - If your sample volume is more then 100 µl then adjust the volume of binding buffer in this step and the bead washing steps
- Add the mix to the washed beads
    - For every 75 µg RNA use 1 mg beads
- Mix on a rotor for 5 min at RT and then place eppie on the magnet stand for 1-2 min
- Remove the supernatant and resuspend in 200 µl washing buffer B. pipet up and odwn several times and put back on the magnetic stand
- Remove all supernatant and repeat the washing step
- Add 20 µl of 10mM Tris Hcl ot the beads. Heat at 75-80°C for 2 minutes and place on the magnetic stand. Transfer the supernatant in a clean eppie
- Immediately continue to cDNA preparation

cDNA preparation

goscript reverse transcription system promega

- maximum 5 µg RNA per reaction
- make the following mix

RNA	Up to 5 µg

Oligo(dT) primers (0.5µg/µl)	1 µl
water	To 5 µl

- Heat the mixture at 70°C for 5 min. immediately put on ice for 5 min
- Prep the reverse transcription mix

Buffer (5x)	4 µl
MgCl2	1.5 µl
Nucleotide mix	1 µl
RNasin	0.5 µl
RTase	1 µl
water	To 15 µl

- Combine the RT mix with the 5 µl RNA mix. Vortex and spin down
- Put in the following program

annealing	25°C	5 min
extention	42°C	60 min
inactivation	70°C	15 min

- Clean up the reaction and measure yield