CAP ANALYSIS GENE EXPRESSION (CAGE) LIBRARY PREPARATION

DAY 1 -

MAKE 70% ETHANOL BEFORE BEGINNING

PUT 1ML CLEAN H_20 TO WATER BATH (37C)

Preparation of RNA

1) Calculate how much µl of RNA samples are needed for 5µg

2) Concentrate the sample to 5.3µl using the concentrator

programme 10, 10 min, no heating, pulse vent yes

check regularly, get to the level of 5.3µl or less (add water if less)

3) Mix with 2.2µl of 210µM (2µg/µl) RT-N15-EcoP primer (7.5µl together)

4) Incubate at 65°C for 5min and then cool on ice immediately (PCR machine MJ3, programme CAGE -> P1)

Reverse transcription of RNA to cDNA/RNA duplex

1) mix the following components

Component	Volume (µl)	Final concentration	9X Volume
PrimeScript buffer	7.5	1X	67.5
dNTPs (10mM each)	1.87	0.5mM each	16.83
Sorbitol (3.3M)/(0.66M) trehalose mix solution	7.5	0.66M/0.132M	67.5
PrimeScript reverse transcriptase (200U/µl)	3.75	750U	33.75
Water	9.38	-	84.42

Total Volume	30	-	-

2) add the enzyme mix solution to the RNA (30µl each, 37.5µl together), carefully mix pipetting on ice (at least 10 times)

3) PCR reaction:

25°C 30s
42°C 30min
50°C 10min
56°C 10min
60°C 10min
4°C forever

(PCR machine MJ3, programme CAGE -> P2)

4) take 2µl aliquot and freeze it at -20°C till the end of the week for QC
- add 2µl of H2O to sample

cDNA/RNA purification with the RNAClean XP kit

1) mix 67.5µl of Agencourt RNAClean XP beads with 37.5µl of RT reaction (1.8x sample ratio), incubate at RT for 30 min, pipette 10 times every 10 minutes

2) Place on the magnetic stand and wait 5min RT

3) carefully aspirate the supernatant – place in a new strip tube on magnetic rack to see if you got any beads in there – if too much, aspirate the supernatant again and wash the beads in the next step

4) wash the beads with 150µl of 70% ethanol (make fresh one every day!) while still in the magnetic stand

5) wash again

6) Add 42µl of pre-heated 37°C water for elution. Pipette 60 times.

7) Incubate the sample at 37°C for 5min minimum (Nature protocol says 10 minutes; PCR programme: P3)

8) Place on the magnetic stand and wait 5min RT

9) transfer the eluent to a new tube (40µl)

10) keep the DNA on ice until the next step

Diol oxidation with NaIO4

1) make solution (during beads incubation) – 250mM NaIO4 (0.053g in 1ml). keep in dark.

2) mix the following reagents on ice and incubate on ice for 45min IN DARK:

Component	Volume(µl)	Final concentration
RNA-cDNA hybrid	40	-
Sodium acetate (1M, pH 4.5)	2	45.7mM
NaIO4, 250mM	2	11mM
Total volume	44	-

3) after incubation, add 2µl of 40% (wt/vol) glycerol and mix thoroughly to stop the oxidation reation. Add 14µl of 1M Tris-HCl (pH 8.5) to bring the pH above 5.6 (total volume 60µl)

cDNA/RNA purification with the RNAClean XP kit

1) mix 108µl of Agencourt RNAClean XP beads with 60µl of diol-oxidation reaction (1.8x sample ratio), incubate at RT for 30 min, pipette 10 times every 10 minutes

2) Place on the magnetic stand and wait 5min RT

3) carefully aspirate the supernatant – place in a new strip tube on magnetic rack to see if you got any beads in there – if too much, aspirate the supernatant again and wash the beads in the next step

4) wash the beads with 150µl of 70% ethanol (make fresh one every day!) while still in the magnetic stand

5) wash again

6) Add 44µl of pre-heated 37°C water for elution. Pipette 60 times.

7) Incubate the sample at 37°C for 5min minimum (Nature protocol says 10 minutes; PCR programme: P3)

8) Place on the magnetic stand and wait 5min RT

9) transfer the eluent to a new tube (42µl)

10) keep the DNA on ice until the next step

TAKE 2µl FOR QC – 40µl total volume

Biotinylation of the RNA diols

1) make 15mM Biotin hydrazide (long arm) solution – dissolve 0.0038g in 675µl of water – continuous mixing is needed, make the solution during the diol-oxidation reaction incubation (vortexing on low setting)

2) Mix the following reagents at least 10X, incubate at 23°C for 16hrs (overnight or so – PCR programme – P4)

Component	Volume (µl)	Final concentration
Purified oxidated cDNA/RNA hybrids	40	-
Sodium citrate (1M, pH 6.0)	4	70mM
Biotin hydrazide (long arm), 15mM	13.5	3.5mM
Total volume	57.5	-

DAY 2

MAKE 70% ETHANOL BEFORE BEGINNING

PUT 1ML CLEAN H_20 TO WATER BATH (37C)

RNase I treatment

1) mix:

Component	Volume (µl)	Final concentration
Biotinylation reaction	57.5	-
Tris-HCl (1M, pH 8.5)	6	86mM
EDTA (0.5M, pH 8.0)	1	7.2mM
RNase ONE ribonuclease (10U/µl)	5	50U
Total volume	69.5	-

2) Incubate in PCR machine using programme P5

37°C 30min
65°C 5min
4°C >2min

During the incubation time, prepare MPG beads Master Mix +1 sample (9) with 100µl (900µl) Streptavidin beads and 1.5µl tRNA (20µg/µl) (13.5µl). Incubate until end of cDNA purification (until needed) on wheel speed 7 in the cold room.

Put the wash buffer 3 at 37°C to dissolve crystallised SDS.

cDNA purification with RNACleanXPkit

1) mix 125.1µl of Agencourt RNAClean XP beads with 69.5µl of RT reaction (1.8x sample ratio), incubate at RT for 30 min, pipette 10 times every 10 minutes

2) Place on the magnetic stand and wait 5min RT

3) carefully aspirate the supernatant – place in a new strip tube on magnetic rack to see if you got any beads in there – if too much, aspirate the supernatant again and wash the beads in the next step

4) wash the beads with 150µl of 70% ethanol (make fresh one every day!) while still in the magnetic stand

5) wash again

6) Add 42µl of pre-heated 37°C water for elution. Pipette 60 times.

7) Incubate the sample at 37°C for 5min minimum (Nature protocol says 10 minutes; PCR programme: P3)

8) Place on the magnetic stand and wait 5min RT

9) transfer the eluent to a new tube (40µl)

10) keep the DNA on ice until the next step

MPG beads preparation

1) Put the tube on a magnetic stand for 2-3 minutes.
2) Remove the supernatant.
3) Wash the beads with 50µl/sample of Wash 1 buffer (450µl)
4) Repeat this one more time.
5) Resuspend the beads in 80µl of Wash 1 buffer per sample (720µl)

Cap Trapping

1) Add 80µl of MPG beads to the 40µl sample, mix by pipetting 10x. incubate at RT for 30 min, pipette 10 times every 5 minutes

2) Place on the magnetic stand and wait 5min RT

3) carefully aspirate the supernatant – place in a new strip tube on magnetic rack to see if you got any beads in there – if too much, aspirate the supernatant again and wash the beads in the next step

4) keep the sample on magnetic stand and wash the beads with 150µl of Wash Buffer 1 (1x), Wash Buffer 2 (1x), Wash buffer 3 (2x), Wash buffer 4 (2x). 6 in total.
At each wash, resuspend the beads and let them separate for 3 min on the magnetic stand before discarding the washing solution.

5) Release – add 60µl of 50mM NaOH solution to the beads and incubate at room temperature for 10 minutes, pipetting every 2-3 minutes. Stand the tube on magnetic stand for 3-5 minutes and then transfer the supernatant to a new strip tube.

6) Add 12µl of 1M Tris-HCl pH 7.0 (Trizma bottle) – cool it on ice – keep on ice before proceeding to the next step.

cDNA purification with AMPure XP kit

1) mix 129.6µl of Agencourt AMPure XP beads with 72µl of RT reaction incubate at RT for 30 min, pipette 10 times every 10 minutes

2) Place on the magnetic stand and wait 5min RT

3) carefully aspirate the supernatant – place in a new strip tube on magnetic rack to see if you got any beads in there – if too much, aspirate the supernatant again and wash the beads in the next step

4) wash the beads with 150µl of 70% ethanol (make fresh one every day!) while still in the magnetic stand

5) wash again

6) Add 42µl of pre-heated 37°C water for elution. Pipette 60 times.

7) Incubate the sample at 37°C for 5min minimum (Nature protocol says 10 minutes; PCR programme: P3)

8) Place on the magnetic stand and wait 5min RT

9) transfer the eluent to a new tube (40µl)

10) keep the DNA on ice until the next step

11) take 5µl aliquot and freeze it at -20°C till the end of the week for QC

Concentration of the cDNA

Concentrate the 35µl of cDNA using centrifugal concentrator at RT to 4µl. (add water if needed)
Check every 7-8 minutes.

Bar-coded 5' linker ligation to the single-stranded cDNA

1) Incubate the cDNA at 65°C for 5 min and cool on ice for 2min (PCR programme P1)
2) At the same time, add 1.5µl of the 5' linker per sample to an empty tube then incubate at 37°C for 5 minutes and cool on ice for 2 min min (PCR programme P6)
3) Add 1µl of the linker to the cDNA (NOTE WHICH ONES ARE WHICH) and 10µl of LigMix Takara. Mix well by pipetting 10x.
4) Incubate at 16°C overnight (PCR programme P7)

DAY 3

MAKE 70% ETHANOL BEFORE BEGINNING

PUT 1ML CLEAN H_20 TO WATER BATH (37C)

Pooling libraries – pool 4 samples together now (2 tubes left)

AMPure purification

1) Pool up to 4 samples in 1 tube (60µl) and wash the 3 empty tubes with 10µl of water. Total volume must be 70µl.
   For 8 samples: you end up with 2 tubes of 70µl.

   Add 126 µl of Agencourt AMPure XP beads to 70µl reaction incubate at RT for 30 min, pipette 10 times every 10 minutes.

2) Place on the magnetic stand and wait 5min RT

3) Carefully aspirate the supernatant – place in a new strip tube on magnetic rack to see if you got any beads in there – if too much, aspirate the supernatant again and wash the beads in the next step

4) wash the beads with 150µl of 70% ethanol (make fresh one every day!) while still in the magnetic stand

5) wash again

6) Add 42µl of pre-heated 37°C water for elution. Pipette 60 times.

7) Incubate the sample at 37°C for 5min minimum (Nature protocol says 10 minutes; PCR programme: P3)

8) Place on the magnetic stand and wait 5min RT

9) transfer the eluent to a new tube (40µl)

AMPure purification again

Pooling libraries step 2 – with first wash step! Only one sample after this.

1) Add 72 µl of Agencourt AMPure XP beads to 40µl reaction incubate at RT for 30 min, pipette 10 times every 10 minutes.

2) Place on the magnetic stand and wait 5min RT

3) Carefully aspirate the supernatant – place in a new strip tube on magnetic rack to see if you got any beads in there – if too much, aspirate the supernatant again and wash the beads in the next step

4) wash the beads with 150µl of 70% ethanol (make fresh one every day!) Mix and wash both tubes with 150µl

5) wash again

6) Add 32.5µl of pre-heated 37°C water for elution. Pipette 60 times.

7) Incubate the sample at 37°C for 5min minimum (Nature protocol says 10 minutes; PCR programme: P3)

8) Place on the magnetic stand and wait 5min RT

9) transfer the eluent to a new tube (30.5µl)

2nd strand synthesis

Component	Volume (µl)	Master Mix (1.5X)
10xLa Taq buffer	5	7.5
25mM MgCl2	5	7.5
2.5mM dNTP	8	12
2nd SOL primer (200ng/µl)	1	1.5
La Taq (5U/µl)	0.5	0.75
Total volume	19.5	

Mix well by pipetting 10x.

Add 19.5µl to the sample and incubate in the PCR machine using programme P8

94°C	3min
42°C	5min
68°C	20min
72°C	2min
4°C	Hold

Antarctic Phosphatase

Add 6µl of 10X Antarctic Phosphatase Reaction Buffer
4µl of Antarctic Phosphatase (5U/µl)

Mix well by pipetting 10x

Incubate in the PCR machine using programme P9

37°C	60min
65°C	5min
4°C	Hold

AMPure purification

1) Add 108 µl of Agencourt AMPure XP beads to 60µl reaction incubate at RT for 30 min, pipette 10 times every 10 minutes.

2) Place on the magnetic stand and wait 5min RT

3) Carefully aspirate the supernatant – place in a new strip tube on magnetic rack to see if you got any beads in there – if too much, aspirate the supernatant again and wash the beads in the next step

4) wash the beads with 150µl of 70% ethanol (make fresh one every day!)

5) wash again

6) Add 32µl of pre-heated 37°C water for elution. Pipette 60 times.

7) Incubate the sample at 37°C for 5min minimum (Nature protocol says 10 minutes; PCR programme: P3)

8) Place on the magnetic stand and wait 5min RT

9) transfer the eluent to a new tube (30µl)

EcoP15I digestion

Component	Master Mix (enough for 10 tubes, µl)
Water	11
10X NEB buffer 3.1	40
100X BSA	4
10X ATP	40
10mM sinefungin	4
EcoP15I (10U/µl)	1
Total	100

Mix well by pipetting 10X.

Add 10µl of the mix to the 30µl of the sample. Incubate 3 hours at 37°C then put on ice for at least 2 minutes using programme P10.

After 3 hours, add 1µl of MgCl2 0.4M, mix well and incubate at 65°C for 20 minutes, then 4°C for 2 minutes using the programme P11.

Meanwhile, during these 20 minutes, make up 3' linker ligation buffer (make fresh every time).

3' linker ligation

5X 3' ligation buffer:

50µl 1M Tris-HCl (pH 7.0)
100µl 10mM ATP
0.5µl 10mg/ml BSA
49.5µl water

Component	Volume (µl)	Master Mix (1.5X)
5X 3'linker ligation buffer	16	24
3' linker (100ng/µl)	1	1.5
T4 DNA ligase (400U/µl)	3	4.5
Water	19	28.5
Total volume	39	

Mix well by pipetting 10X.

Mix 39µl of 3' linker mix with 41µl of the sample. Incubate at 16°C for 14-16 hours (overnight) using the programme P7.

DAY 4

Preparation of Streptavidin beads

Put the Wash Buffer 3 in the 37°C water bath

Mix 15µl of MPG Streptavidin beads with 1.5µl of tRNA (20µg/µl). Incubate on ice for 30min tapping every 5 minutes.

Stand on the magnetic stand for 5 minutes. Remove supernatant, wash the beads with 75µl of Wash Buffer 1 TWICE.

After 2 washes, resuspend the beads in 37.5µl of Wash Buffer 1.

Purification from the 3'linker by beads

1) Add 25µl of prepared MPG beads with tRNA to 80µl of the sample from yesterday, mix well. Incubate at room temperature for 30 minutes, mixing every 5 minutes.

2) Place on the magnetic stand and wait 5min RT

3) carefully aspirate the supernatant – place in a new strip tube on magnetic rack to see if you got any beads in there – if too much, aspirate the supernatant again and wash the beads in the next step

4) keep the sample on magnetic stand and wash the beads with 150µl of Wash Buffer 1 (1x), Wash Buffer 2 (1x), Wash buffer 3 (2x), Wash buffer 4 (2x). 6 in total.
At each wash, resuspend the beads and let them separate for 3 min on the magnetic stand before discarding the washing solution.

5) Final wash – 50µl of RNase/DNase free water. Be very quick, keep the water in a tube in case of anything happening.

6) Resuspend the beads in 20µl of water. You can pause here by putting them to 4°C or -20°C freezer. These beads are used as PCR template.

Bring Agilent DNA1000 Tapestation kit to room temperature.

PCR cycle check

Prepare 1 PCR reaction per pooled library.

water	32.5µl
5XHF buffer	10µl
2.5mM dNTP	4µl
100µM F primer	0.5µl
100µM R primer	0.5µl
Phusion (2U/µl)	0.5µl
Total	48µl

Mix well by pipetting 10X.

Add 2µl of template beads to the mix, total volume 50µl. Mix well. Do not spin down (beads would go to the bottom of the tube).

Incubate in PCR machine using programme PCR21 following the instructions below.

98°C	30s	
98°C	10s	20 cycles
60°C	10s	
4°C	Hold	

During the PCR, pause the machine after 8, 10, 12, 14 and 16 cycles – take out 4µl quickly out of the tube and store in a marked tube. This is used for Tapestation to determine if CAGE worked and what is the optimum PCR cycle number.

Determine the optimum PCR cycle number

Prepare the ladder (always first in a strip tube) by mixing 3µl D1000 Sample Buffer with 1µl D1000 ladder.

Prepare the samples by mixing 3µl D1000 sample buffer with 1µl DNA sample.

Spin down, vortex using IKA vortexer at 2000rpm for 1 minute.

Spin down again, place the samples and screentape in Tapestation.

Decide on the best number of cycles based on the concentration of product (~100bp) and linker (~50bp). You want one big peak at ~100, not too many bigger peaks and a small linker peak. Usually the number of cycles is 10-11.

Bulk PCR

Prepare 6 PCR reaction tubes for bulk PCR.

		Master mix (6.5X, µl)
water	32.5µl	211.25
5XHF buffer	10µl	65
2.5mM dNTP	4µl	26
100µM F primer	0.5µl	3.25
100µM R primer	0.5µl	3.25
Phusion (2U/µl)	0.5µl	3.25
Total	48µl	

Mix well by pipetting 10X.

Add 2µl of template PCR beads to the 48µl master mix.

Mix well by pipetting 10X.

Incubate in the PCR machine using the optimal number of cycles using programme PCR21 (same as last time).

You can pause here, keeping the product on ice.

Exonuclease I treatment

Pool three reactions together, getting 2 tubes in total. Add 1µl of Exonuclease I (20U/µl) to each of the two 150µl PCR solutions. Mix by pipetting on ice and incubate at 37°C for 30min using the programme P13.

Minelute PCR Purification kit

Purify 151µl of Exonuclease I- treated CAGE tags using Minelute PCR purification kit, following the manufacturer's instructions. At the end, elute in 12µl+12µl EB buffer. Pool these two samples into 1 tube.

Concentration check

Use 2µl of the eluent to check the concentration with the Agilent Tapestation DNA1000 kit as previously.