AQUA-FAANG - Standard Operating Protocol - CAGE (Cap Analysis of Gene Expression) Protocol in carp embryos

Overview

CAGE (cap analysis of gene expression) allows for precise mapping of transcription start sites in eukaryotic genomes, based on sequencing the 5'-ends of capped RNAs. This protocol is based on the Murata et al., (2024) methods with adaptations and comments from Dr Bojan Žunar, Dr Damir Baranašić and Dr Yavor Hadzhiev. Throughout the protocol, the total cellular RNA is first reversely transcribed using random primers to produce RNA–DNA duplexes. The reaction mixture is then treated with sodium periodate, which oxidises diols present exclusively on the last 3'-ribose moiety in all RNA molecules and on the 7-methyl guanylate cap of capped RNA molecules. Oxidised diols are biotinylated, and the mixture treated with RNaseONE, which degrades ssRNA. The remaining biotinylated 5'-ends are pulled from the mixture with streptavidin beads, their single-stranded cDNA recovered, and their ssRNA degraded with RNase H. Finally, the cDNA is ligated to specific 5'- and 3'-linkers flanked with Illumina adaptors and sequenced.

Equipment/Kits

- Thermocycler with heated lid
- Magnetic stand (for 1.5 ml and PCR tubes)
- Non-magnetic stand
- Eppendorf Concentrator plus (vacuum concentrator)
- Centrifuge for 1.5 ml tubes
- Low-binding 1.5 ml Eppendorf tubes
- PCR tubes
- SuperScript III Reverse Transcriptase
- RNAClean XP beads (Beckman Coulter)
- AMPure XP beads (Beckman Coulter)
- Dynabead M-270 streptavidin beads
- RNaseONE (10 U/µl, Promega)
- RNase H (60 U/µl, Takara)
- Mighty Mix DNA Ligase (Takara)
- NEBNext Ultra II Q5 Master Mix (NEB)
- Qubit device and reagents (High Sensitivity)

- Bioanalyzer or TapeStation

Solutions/Reagents

- RNase-free H_2O
- RT_TCT-N6 primer (2.5 mM)
- 1st strand buffer (5x conc.)
- DTT (0.1 M)
- dNTPs (10 mM each)
- Trehalose (0.7 M)/sorbitol solution (3.25 M)
- Sodium periodate ($NaIO_4$) – prepare fresh at 250 mM
- Biotin hydrazide – prepare fresh at 20 mM
- Sodium acetate buffer (1 M, pH 4.5, RNase-free)
- Sodium acetate buffer (1 M, pH 6.0, RNase-free)
- Glycerol (40%)
- Tris-HCl (1 M, pH 8.5)
- S. cerevisiae tRNA (10 µg/µl)
- Ice-cold 80% ethanol
- TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA)
- NaCl (1 M and 5 M stock)
- EDTA (0.5 M, pH 8.0)
- Tween 20
- 5'- and 3'-oligonucleotide linkers (see Step 16)
- UDI Illumina adapter primers (UDI001–UDI024)

DAY 1

1. Reverse Transcription

1.1. Anneal the random primer to the total RNA. Mix the following components:

Component	Volume (µl)
RT_TCT-N6 primer (2.5 mM)	1.0
RNA (5–6.5 µg/sample)	up to 9.0
RNase-free H_2O	as needed
Total	10.0

1.2. Mix by pipetting. Incubate in a thermocycler at 65°C for 5 min with lid heated to 105°C. Place on ice for 2 min.

1.3. Prepare the SuperScript III master mix:

Component	Volume (µl)
RNase-free H_2O	6.1
1st strand buffer (5x conc.)	7.6
DTT (0.1 M)	1.9
dNTPs (10 mM each)	1.0
Trehalose (0.7 M)/sorbitol solution (3.25 M)	7.6
SuperScript III reverse transcriptase (200 U/µl)	3.8

1.4. Mix randomly primed RNA with SuperScript III master mix:

Component	Volume (µl)
Randomly primed RNA (step 1.2)	10.0
SuperScript III master mix (step 1.3)	28.0
Total	38.0

1.5. Mix by pipetting and incubate at 25°C for 30 s, then at 50°C for 60 min. Store at 4°C for up to several minutes.

Prepare the sodium periodate ($NaIO_4$) solution (250 mM) and biotin hydrazide solution (20 mM) at this stage, as they are required in steps 3 and 5, respectively.

2. Purification with RNAClean XP Beads

2.1. Pre-warm the RNAClean XP beads to room temperature.

2.2. Add RNAClean XP beads to each sample at a 1:1.8 sample-to-beads ratio and mix by pipetting:

Component	Volume (µl)
Reversely transcribed sample (step 1.5)	38.0
RNAClean XP beads	68.4
Total	110.0

2.3. Place on a non-magnetic stand and incubate for 5 min at room temperature.

2.4. Transfer to a magnetic stand, immobilise with transparent tape, and incubate for 5 min at room temperature.

2.5. Leaving the samples on the magnetic stand, carefully remove and discard the supernatant without disturbing the beads.

2.6. Leaving the samples on the magnetic stand, wash beads twice with 200 µl of ice-cold 80% EtOH, removing each wash without disturbing the beads.

2.7. Remove samples from the magnetic stand and air-dry the beads for several seconds. Elute nucleic acids with 42 µl of RNase-free water pre-heated to 37°C by pipetting 60 times, followed by incubation at 37°C for 5 min.

2.8. Place on the magnetic stand, immobilise with transparent tape and incubate for 5 min at room temperature. Transfer 40 µl of the supernatant (containing eluted cDNA) to a new set of PCR tubes. Do not disturb the beads.

3. Oxidation of Diols

$NaIO_4$ requires an acidic medium to oxidise diols. Mix the purified RT sample with sodium acetate buffer:

3.1. Buffer the purified RT sample:

Component	Volume (µl)
Purified RT sample (step 2.8)	40.0
Sodium acetate buffer (1 M, pH 4.5)	2.0
Total	42.0

3.2. Add $NaIO_4$ and mix by pipetting:

Component	Volume (µl)
Buffered RT sample (step 3.1)	42.0
$NaIO_4$ (250 mM, RNase-free)	2.0
Total	44.0

3.3. Incubate on ice for 45 min in the dark.

3.4. Stop the oxidation by adding glycerol and raising the pH:

Component	Volume (µl)
$NaIO_4$ reaction mixture (step 3.3)	44.0
Glycerol (40%)	2.0
Tris-HCl (1 M, pH 8.5)	14.0
Total	60.0

3.5. Mix by pipetting. Proceed immediately to purification.

4. Purification with RNAClean XP Beads

4.1. Repeat steps 2.1–2.8 using the oxidised sample (step 3.5) with a 1:1.8 sample-to-beads ratio (60 µl sample + 108 µl beads). Elute the nucleic acids from the beads with a 42 µl of RNase-free water pre-heated to 37°C. Transfer 40 µl of the eluate to new PCR tubes.

5. Biotinylation

5.1. Prepare sodium acetate buffer (pH 6.0, 1 M, RNase-free) by mixing:

Component	Volume (µl)
Sodium acetate buffer (pH 5.2, 3 M)	500.0
Sodium acetate buffer (pH 7.0, 3 M)	500.0
RNase-free H_2O	2000.0
Total	3000.0

5.2. Adjust the pH of the purified oxidised sample:

Component	Volume (µl)
Purified oxidised sample (step 4.1)	40.0
Sodium acetate buffer (1 M, pH 6.0)	4.0
Total	44.0

5.3. Add biotin hydrazide and mix:

Component	Volume (µl)
Buffered oxidised sample (step 5.2)	44.0
Biotin hydrazide (20 mM, pH 6.0)	2.0
RNase-free H_2O	2.0
Total	48.0

5.4. Incubate at 23°C for 2 h in a thermocycler, in the dark.

6. Purification with RNAClean XP Beads

6.1. Repeat steps 2.1–2.8 using the biotinylated sample (step 5.4) and **adding isopropanol (48 µl sample + 12 µl isopropanol + 108 µl beads)**. Elute the nucleic acids from the beads with a 42 µl of RNase-free water pre-heated to 37°C. Transfer 40 µl of the eluate to new PCR tubes.

6.2. Store at −80°C.

END OF DAY 1

DAY 2

7. Digestion with RNaseONE

7.1. Mix by pipetting:

Component	Volume (µl)
Purified biotinylated sample (step 6.1)	40.0
RNaseONE buffer (10x conc.)	4.5
RNaseONE (10 U/µl)	0.5
Total	45.0

7.2. Incubate at 37°C for 30 min. If needed, store at 4°C for up to several hours.

8. Preparation of Dynabead M-270 Streptavidin Beads

8.1. To coat the beads with tRNA, mix the following in a single 1.5 ml low-binding microtube:

Component	Volume (µl)
Dynabead M-270 streptavidin beads	30.0
S. cerevisiae tRNA (10 µg/µl)	0.8
Total	30.8

8.2. Place on ice for 30 min, mixing by pipetting every 5 min.

Prepare washing buffers A, B and C fresh during the incubation (see below). For 9 samples: 2565 µl Buffer A, 1350 µl Buffer B, and 1350 µl Buffer C are required.

8.3. Prepare washing buffer A (4.5 M NaCl, 50 mM EDTA pH 8.0, 0.1% Tween 20):

Component	Volume (µl)
NaCl (5 M)	4495.0
EDTA (0.5 M, pH 8.0)	500.0
Tween 20	5.0
Total	5000.0

8.4. Prepare washing buffer B (10 mM Tris-HCl pH 8.5, 1 mM EDTA pH 8.0, 0.5 M sodium acetate pH 6.1, 0.1% Tween 20):

Component	Volume (µl)
Tris-HCl (1 M, pH 8.0)	50.0
EDTA (0.5 M, pH 8.0)	10.0
Sodium acetate buffer (1 M, pH 6.0)	2500.0
Tween 20	5.0
RNase-free H_2O	2435.0
Total	5000.0

8.5. Prepare washing buffer C (0.3 M NaCl, 1 mM EDTA pH 8.0, 0.1% Tween 20):

Component	Volume (µl)
NaCl (5 M)	300.0
EDTA (0.5 M, pH 8.0)	10.0
Tween 20	5.0
RNase-free H_2O	4685.0
Total	5000.0

8.6. Place the 1.5 ml microtube on a dedicated magnetic stand, immobilise it with transparent scotch tape, and incubate for 3 min at room temperature. Leaving the tube immobilised on the magnetic stand, carefully remove and discard the supernatant, using a pipette, without disturbing the beads. Remove the tube from the magnetic stand, add 135 µl of washing buffer A, and mix by pipetting.

8.7. Place the 1.5 ml microtube on a dedicated magnetic stand, immobilise it with transparent scotch tape, and incubate for 3 min at room temperature. Leaving the tube immobilised on the magnetic stand, carefully remove and discard the supernatant, using a pipette, without disturbing the beads. Remove the tube from the magnetic stand and resuspend beads in 945 µl of washing buffer A with S. cerevisiae tRNA (10 µg/µl) and mix by pipetting.

9. Cap-Trapping

9.1. Combine the RNaseONE-treated sample with streptavidin and tRNA-coated beads:

Component	Volume (µl)
RNaseONE-treated sample (step 7.2)	45.0
Streptavidin/tRNA-coated M-270 beads (step 8.7)	105.0
Total	150.0

9.2. Incubate at 37°C for 30 min in a thermocycler. Mix by pipetting every 10 min to prevent bead precipitation.

9.3. Place on the magnetic stand, immobilise with transparent tape, and incubate for 5 min. Remove and discard the supernatant without disturbing the beads.

9.4. Remove the tube from the magnetic stand. Wash with 150 µl washing buffer A. Place on magnetic stand, incubate 5 min, and discard supernatant.

9.5. Remove the tube from the magnetic stand. Wash with 150 µl washing buffer B (pre-heated to 37°C). Place on magnetic stand, incubate 5 min, and discard supernatant.

9.6. Remove the tube from the magnetic stand. Wash with 150 µl washing buffer C (pre-heated to 37°C). Place on magnetic stand and incubate 5 min.

9.7. Prepare the elution buffer:

Component	Volume (µl)
RNaseONE buffer (10x conc.)	6.5
RNase-free H_2O	58.5
Total	65.0

9.8. Discard supernatant from step 9.6. Proceed immediately to the next step to prevent over-drying of the beads.

10. Elution of cDNA

10.1. Add 35 µl of elution buffer (step 9.7) to each bead sample and mix by pipetting.

10.2. Incubate at 95°C for 5 min, then place on ice for 2 min.

10.3. Place on the magnetic stand, immobilise with transparent tape, incubate for 5 min, and carefully transfer 35 µl of supernatant to new PCR tubes.

10.4. Perform a second elution: add 30 µl of elution buffer to the beads and mix by pipetting. Incubate at 95°C for 2 min, place on ice for 2 min, place on the magnetic stand for 5 min, then transfer 30 µl of supernatant to the same PCR tubes. Final volume: 65 µl.

11. Digestion with RNase H and RNaseONE

11.1. Prepare the RNase master mix:

Component	Volume (µl)
RNase-free H_2O	2.4
RNaseONE buffer (10x conc.)	0.5
RNase H (60 U/µl, Takara)	0.1
RNaseONE (10 U/µl, Promega)	2.0
Total	5.0

11.2. Add 5 µl of RNase master mix to each 65 µl sample. Mix by pipetting.

11.3. Incubate at 37°C for 15 min. Store at 4°C for up to several hours.

12. Purification with AMPure XP Beads

12.1. Add AMPure XP beads at a 1:1.8 ratio (70 µl sample + 126 µl beads). Repeat steps 2.3–2.8, eluting in 42 µl of RNase-free water pre-heated to 37°C. Transfer 40 µl of eluate to new PCR tubes.

13. Second Digestion with RNaseONE

13.1. Repeat step 7.1 using the cap-trapped cDNA from step 12.1. Incubate at 37°C for 30 min.

14. Purification with AMPure XP Beads (post-second RNaseONE)

14.1. Add AMPure XP beads at a 1:1.8 ratio (45 µl sample + 81 µl beads). Repeat steps 2.3–2.8, eluting in 42 µl of RNase-free water pre-heated to 37°C. Transfer 40 µl of eluate to new PCR tubes.

> *Quality control note: At this stage 0.15–0.20 ng/µl of cDNA is expected. NanoDrop is not suitable below 10 ng/µl. Bioanalyzer/TapeStation may be used for QC, or qPCR with ACT1B and 18S rRNA primers to assess mRNA:rRNA ratio.*

15. Concentration of cDNA with Vacuum Concentrator

The ultimate goal of day 2 is to add 5'-linkers to the 5'-end of the cDNA molecules. However, as the ligation of double-stranded linkers to single-stranded cDNA molecules is less-than-efficient, the ligation mixture needs to be very dense, i.e. it allows for a minimal cDNA volume. For this purpose, before preparing the ligation mixture, we concentrate cDNA using a vacuum concentrator.

15.1. Transfer the entire purified cDNA sample (step 14.1) to low-binding 1.5 ml Eppendorf tubes.

15.2. Concentrate using an Eppendorf Concentrator plus: 40 min evaporation time, brake off, 30°C, V-AQ mode.

15.3. Resuspend the pellet in 7 µl of RNase-free H_2O.

16. Annealing of 5’- and 3’-Linkers

16.1. Dissolve all oligonucleotides to 1 mM in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

16.2. Prepare the 5’-oligonucleotide annealing reaction:

Component	Volume (µl)	Final concentration
5pUP-GN5 (1 mM) or 5pUP-N6 (1 mM)	1.0	100 µM
5pDN (1 mM)	1.0	100 µM
NaCl (1 M)	1.0	100 mM
TE buffer	7.0	—

Component	Volume (µl)	Final concentration
Total	10.0	

16.3. Prepare the 3’-oligonucleotide annealing reaction:

Component	Volume (µl)	Final concentration
3pUP-N3AGA (1 mM)	1.0	100 µM
3pDN (1 mM)	1.0	100 µM
NaCl (1 M)	1.0	100 mM
TE buffer	7.0	—
Total	10.0	

16.4. Anneal in the thermocycler using the following programme:

Temperature	Duration
95.0°C	5 min
ramp −0.1°C/s → 83.0°C	
83.0°C	5 min
ramp −0.1°C/s → 71.0°C	
71.0°C	5 min
ramp −0.1°C/s → 59.0°C	
59.0°C	5 min
ramp −0.1°C/s → 47.0°C	
47.0°C	5 min
ramp −0.1°C/s → 35.0°C	
35.0°C	5 min
ramp −0.1°C/s → 11.0°C	
11.0°C	5 min

16.5. Spin down the tubes. Prepare 10 µM working solutions:

5’-linker mix:

5pUP-GN5+5pDN (100 µM)	10 µl
5pUP-N6+5pDN (100 µM)	2.5 µl
NaCl (0.1 M)	112.5 µl

Total	125 µl

3’-linker mix:

3pUP-N3AGA+3pDN (100 µM)	10 µl
NaCl (0.1 M)	90 µl
Total	100 µl

Store working solutions at −20°C.

17. Ligation of 5’-Linkers to Single-Stranded cDNA

17.1. Disrupt cDNA secondary structures: incubate at 95°C for 5 min, then immediately transfer to ice for 2 min.

17.2. Disrupt 5’-linker dimers (10 µM): incubate at 60°C for 5 min, then immediately transfer to ice for 2 min.

17.3. Assemble the ligation reaction:

Component	Volume (µl)
Purified cDNA (step 15.3)	7.0
5’-linker (10 µM, step 16.5)	1.0
Mighty Mix Ligation (Takara)	16.0
Total	24.0

17.4. Incubate at 16°C for 16 h.

END OF DAY 2

DAY 3

18. Purification with AMPure XP Beads – 1st Purification (post-5' ligation)

18.1. Add AMPure XP beads at a 1:1.8 ratio (24 µl sample + 43.2 µl beads). Repeat steps 2.3–2.8, eluting in 42 µl of RNase-free water pre-heated to 37°C. Transfer 40 µl of eluate to new PCR tubes.

19. Purification with AMPure XP Beads – 2nd Purification

19.1. Add AMPure XP beads at a 1:1.8 ratio (40 µl sample + 72 µl beads). Repeat steps 2.3–2.8, eluting in 42 µl of RNase-free water pre-heated to 37°C. Transfer 40 µl of eluate to new PCR tubes.

20. Concentration of cDNA with Vacuum Concentrator

20.1. Repeat steps 15.1–15.3 using the purified cDNA with added 5'-linkers (step 19.1). On Eppendorf Concentrator plus, use the following parameters: 40 min evaporation time, brake off, the temperature of 30°C, and V-AQ mode. Resuspend the pellet in 7 µl of RNase-free H2O.

21. Ligation of 3'-Linkers to Single-Stranded cDNA

21.1. Disrupt cDNA secondary structures: incubate at 95°C for 5 min, then immediately transfer to ice for 2 min.

21.2. Disrupt 3'-linker dimers (10 µM): incubate at 60°C for 5 min, then immediately transfer to ice for 2 min.

21.3. Assemble the ligation reaction:

Component	Volume (µl)
Purified cDNA (step 20.1)	7.0
3'-linker (10 µM, step 16.5)	1.0
Mighty Mix Ligation (Takara)	16.0
Total	24.0

21.4. Incubate at 16°C for 16 h.

END OF DAY 3

DAY 4

22. Purification with AMPure XP Beads – 1st Purification (post-3' ligation)

22.1. Add AMPure XP beads at a 1:1.8 ratio (24 µl sample + 43.2 µl beads). Repeat steps 2.3–2.8, eluting in 42 µl of RNase-free water pre-heated to 37°C. Transfer 40 µl of eluate to new PCR tubes.

22.2. Incubate tubes at 95°C for 5 min, then place on ice for 2 min.

23. Purification with AMPure XP Beads – 2nd Purification

23.1. Add AMPure XP beads at a 1:1.8 ratio (40 µl sample + 72 µl beads). Repeat steps 2.3–2.7 but **elute in 20 µl of RNase-free water pre-heated to 37°C**. Transfer 20 µl of eluate using a standard (not multichannel) pipette to recover every microlitre.

24. Second-Strand Synthesis and Introduction of Illumina TruSeq Adaptors

24.1. Prepare Illumina adapter mixes (one unique dual index per sample):

Component	Volume (µl)	Final concentration
UDI00##_I5 (20 µM)	20.0	10 µM
UDI00##_I7 (20 µM)	20.0	10 µM
Total	40.0	

24 unique primer pairs are available (UDI001–UDI024), enabling pooling of up to 24 samples per Illumina lane. Adapter mixes can be stored at −20°C for several months.

24.2. Assemble the PCR reaction:

Component	Volume (µl)
Purified cDNA (step 23.1)	20.0
Illumina adapter mix (10 µM)	5.0
NEBNext Ultra II Q5 Master Mix (NEB)	25.0
Total	50.0

24.3. Run the following PCR programme (second-strand synthesis + 5 PCR cycles):

Step	Temperature	Duration
Initial denaturation	98°C	30 s
Denaturation × **6 cycles**	98°C	10 s
Extension	65°C	2 min

Step	Temperature	Duration
Final extension	65°C	5 min
Hold	4°C	Indefinite

24.4. Store at 4°C for up to several hours.

25. Final Purification with AMPure XP Beads

25.1. Add AMPure XP beads at a 1:1 ratio (50 µl sample + 50 µl beads). Repeat steps 2.3–2.7, **eluting in 20 µl of RNase-free water pre-heated to 37°C.** Transfer 20 µl of eluate using a standard (not multichannel) pipette.

25.2. Store at −20°C.

26. Sequencing

26.1. Submit 3 µl of each sample for QC (Qubit + Bioanalyzer/TapeStation).

26.2. Using the Illumina Pooling Calculator, calculate the volume of each sample required for equimolar pooling.

26.3. Pool calculated volumes into a single low-binding 1.5 ml microtube and submit to the genomics facility together with adapter barcode references.