ATAC-seq standard protocol from cryopreserved cells

For any questions regarding this protocol, feel free to send an email to info@deep-dv.org

Precheck

- Cells should be cryopreserved with standard freezing medium containing 10% DMSO and stored at -80°C until processed. Using frozen cell pellets (without DMSO) is **not** recommended and leads to cell death and strong background signal of free DNA
- get ice, cool centrifuge to 4°C
- buffers needed fresh:
    - **Wash buffer:** PBS + 0.04% final BSA
    - **DNAse buffer:** PBS +0.04% BSA + 3mM MgCl2
    - **RSB** (2 ml per sample)
    - **NTD buffer** (50 µl per sample)
    - **RSB-T buffer** (1 ml per sample)
    - **Tn5 mix** – add Tn5 at the last minute
- medium (depending on cells) (warm up)
- PBS
- Trypsin-EDTA (0.25%) (Gibco™ 25200056)
- 100x DNase (stock conc. 20k U/ml) (Worthington, LS002006)
- Transposase stock 2,5 µM (custom made)
- Digitionin (Promega, G9441, supplied stock 2% in DMSO, dilute 1:1 with Water to make 1% stock, store at -20°C)
- -Tween-20 (stock 10%) (Sigma, 11332465001)
- -NP40 (stock 10%) (VWR, PIER85124)
- Zymo DNA Clean and Concentrator-5 Kit (Zymo Research, D4014)

Prepare cells

- Thaw cryotubes and transfer cells to 15 ml tube in 10 ml prewarmed medium
- Centrifuge at 300 rcf for 5min
- Remove supernatant without disturbing the pellet
- Resuspend in 1ml DNAse buffer
- Rinse 15 ml tube with 0.5 ml DNAse buffer and add to 2ml tube
- Add 15 µl DNase (200 U/ml) per ml medium to the cells. Incubate for 5 min on ice
- Add 4.5 ul EDTA 0.5M (1.5mM final)
- Centrifuge at 500 rcf at 4°C for 10 min
- Add 1 ml cold Wash buffer (PBS + 0.04% BSA)
- Count the cells
- Aliquot 100.000 cell samples in Eppis (**no** low-binding tubes), spin down cells, 10min 500 rcf 4°C
- Resuspend in 1 ml cold RSB buffer
    - Optional: Keep rest of the cells for input control f.ex. to observe infection rate→ spin down, remove supernatant, store pellet at -20°C

ATAC-seq

- Centrifuge at 500 g, 4°C, 5 min. Carefully remove supernatant in two steps
- Add 50µl cold ATAC-NTD buffer, pipette up and down 3x, incubate on ice 3 min
- Wash out lysis buffer with 1 ml cold ATAC-T, invert tube 3x to mix

- Centrifuge at 500 g, 4 °C, 10 min. Carefully remove supernatant in two steps
- Add Tn5 to Transposition mix. Resuspend pellets in 50 µl Transposition mix (pipette up and down 6x) and incubate at 37°C, 1000 rpm in a thermomixer for 30 min
- Stop Tn5 reaction by adding 250 µl DNA binding buffer from Zymo DNA Clean and Concentrator-5 Kit (cat#D4014)
    - Briefly mix sample by vortexing
    - Transfer mixture to Spin column in collection tube (remove cap)
    - Centrifuge (10.000g) 30 s, discard flow-through
    - add 200 µl DNA wash buffer, centrifuge 30 s, discard flow-through. Repeat wash
    - add 21 µl DNA Elution buffer, incubate 1 min, transfer to new tube
    - centrifuge 30 s to elute DNA

Store at -20°C

Buffers

RSB	**50 ml**
1M Tris-HCl pH 7.4 (final 10 mM)	500 µl
5M NaCl (final 10 mM)	100 µl
1M $MgCl_2$ (final 3 mM)	150 µl
H_2O	49,25 ml

fresh **ATAC-T**	**1 sample**
RSB	990 µl
10% Tween-20 (final 0.1%)	10 µl

2x TD buffer	**50 ml**
1M Tris HCl 7,6 pH (final 20 mM)	1 ml
1M $MgCl_2$ (final 10mM)	500 µl
Dimethyl Formamide (final 20%)	10 ml
H_2O to	50 ml

fresh **ATAC-NTD**	**1 sample**
RSB	48,5
10% NP40 (final 0.1%)	0,5
10 % Tween 20 (final 0.1%)	0,5
1 % Digitonin (final 0.01%)	0,5

Transposition mix	**1 sample**
2x TD buffer	25 µl
PBS	16,5 µl
H_2O	5.5 µl
1 % Digitonin (final 0.01%)	0,5 µl
10 % Tween 20 (final 0.1%)	0,5 µl
2.5µM Transposase (final 100 nM)	2 µl

Library-Preparation

- Use all 20 ul of product in the following PCR to introduce Illumina-Sequencing compatible barcodes.
- Follow the Pipetting scheme below using NEBNext Ultra II Q5 2× Master Mix (New England Biolabs, cat. no. M0544S) and a unique combination of Ad1 and Ad2 per sample for library preparation:
  - Run 5 initial cycles of Adapter-PCR on a thermo cycler:

Adapter-PCR	
25 uM Primer Ad1	2.5 ul
25 uM Primer Ad2	2.5 ul
2x NEBNext Master Mix	25 ul
Transposed Sample	20 ul

Cycling Conditions	
72°C	5 min
98°C	30 sec
Then 5 cycles of:	
98°C	10 sec
63°C	30 sec
72°C	1 min
4°C	Hold at

  - Determine additional PCR cycles (meanwhile, store Adapter-PCR reaction mix on ice)

Option 1: qPCR

- Using 5 ul (10%) of the pre-amplified mixture, run a 15 ul qPCR
- The number of additional cycles X can be calculated by plotting the raw fluorescence against the cycle number. Estimate the qPCR cycle number at the position, where the raw fluorescence has reached 1/3 of its maximum. This equals the number of additional cycles.

Cycling Conditions	
Hot start	
98°C	30 sec
Then 20 cycles of:	
98°C	10 sec
63°C	30 sec
72°C	45 sec
Hold at 4°C	

qPCR Reaction	
Sterile water	3.76 ul
25 uM Primer Ad1	0.5 ul
25 uM Primer Ad2	0.5 ul
2x SYBR Green (in DMSO)	0.24 ul
2x NEBNext Master Mix	5 ul
Pre-Amplified Sample	5 ul

Option 2: Qubit concentration

- Use 2 µL of the Adapter-PCR mix after initial 5 cycles and quantify concentration by Qubit measurement (DNA HS Assay).
- Apply the following formular to determine additional cycles X (round to nearest whole integer):

$$X = -5.7 * log10\left(Qubit\ conc.\left[\frac{ng}{\mu l}\right]\right) + 6.7$$

After applying Option 1 or Option 2 continue:

- Use the remaining amount of Adapter-PCR reaction mix to run the following parameter on a thermo cycler:

Cycling Conditions	
98°C	30 sec
Then X cycles of:	
98°C	10 sec
63°C	30 sec
72°C	45 sec
Incubation Extension 72 °C	3 min
Hold at 4°C	

- Purify and size-select the amplified library with AMPure beads (or similar):
  - Incubate beads 30 min at room temperature.
  - Fill PCR-mix with nuclease-free water to 50 µl total volume.
  - Add 27,5 µL of magnetic beads and mix thoroughly by pipetting.
  - Incubate at room temperature for 5 min.
  - Spin briefly and place the samples on a magnetic stand for 5 min at RT.
  - Collect the clear supernatant in a fresh tube (~75 µl).
  - Discard beads (large fragments are binding to those).
  - Add fresh magnetic beads to the tube with collected supernatant targeting a final ratio of 1.8X (62,5 µl fresh beads to the full volume of the supernatant from previous step) and mix thoroughly by pipetting.
  - Incubate at room temperature for 5 min.
  - Discard the clear supernatant.
  - Add 200 µL freshly prepared 80 % ethanol without disturbing the beads.
  - Incubate for 30 sec at RT.
  - Carefully remove and discard the supernatant without disturbing the beads.
  - Repeat the last three steps.
  - Air-dry the beads for 5 min at RT.
  - Resuspend the dried beads completely in 16 µL EB Buffer with 0,1 % Tween-20 or nuclease-free water.
  - Incubate for 2 min at RT.
  - Spin briefly and place the samples on a magnetic stand.
  - Gently transfer 15 µL of clear sample to a new 1,5 mL tube.
  - Store the samples at -20°C or directly check size and concentration.

- As an alternative to this double size-selection, a one-sided size-exclusion of potential adapter peaks can be applied (dependent on the transposase reaction

the number of large fragments is lower and a double size-selection is not needed). In this case, perform a regular purification starting with 1.8X beads.

- Quality control the library by performing a TapeStation or Bioanalyzer assay, expecting the typical nucleosome pattern.
- Adjust concentration per library and pool dependent on the intended sequencing system. ATAC-libraries should be sequenced in a paired-end mode.

Library Barcode Adapter (from Grandi et al. 2022, doi.org/10.1038/s41596-022-00692-9)

Custom Barcodes Adapter 1 (index i5):	
Adapter Name	Adapter Sequence
Ad1.1	AATGATACGGCGACCACCGAGATCTACACTAGATCGCTCGTCGGCAGCGTCAGATG TGTAT
Ad1.2	AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGCAGCGTCAGATG TGTAT
Ad1.3	AATGATACGGCGACCACCGAGATCTACACTATCCTCTTCGTCGGCAGCGTCAGATG TGTAT
Ad1.4	AATGATACGGCGACCACCGAGATCTACACAGAGTAGATCGTCGGCAGCGTCAGAT GTGTAT
Ad1.5	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGTCGTCGGCAGCGTCAGAT GTGTAT
Ad1.6	AATGATACGGCGACCACCGAGATCTACACACTGCATATCGTCGGCAGCGTCAGATG TGTAT
Ad1.7	AATGATACGGCGACCACCGAGATCTACACAAGGAGTATCGTCGGCAGCGTCAGAT GTGTAT
Ad1.8	AATGATACGGCGACCACCGAGATCTACACCTAAGCCTTCGTCGGCAGCGTCAGATG TGTAT
Ad1.9	AATGATACGGCGACCACCGAGATCTACACTGGAAATCTCGTCGGCAGCGTCAGATG TGTAT
Ad1.10	AATGATACGGCGACCACCGAGATCTACACAACATGATTCGTCGGCAGCGTCAGATG TGTAT
Ad1.11	AATGATACGGCGACCACCGAGATCTACACTGATGAAATCGTCGGCAGCGTCAGATG TGTAT
Ad1.12	AATGATACGGCGACCACCGAGATCTACACGTCGGACTTCGTCGGCAGCGTCAGATG TGTAT
Ad1.13	AATGATACGGCGACCACCGAGATCTACACTTTCTAGCTCGTCGGCAGCGTCAGATG TGTAT
Ad1.14	AATGATACGGCGACCACCGAGATCTACACTAACCAAGTCGTCGGCAGCGTCAGATG TGTAT
Ad1.15	AATGATACGGCGACCACCGAGATCTACACGTGTATCGTCGTCGGCAGCGTCAGATG TGTAT
Ad1.16	AATGATACGGCGACCACCGAGATCTACACTCCATCAATCGTCGGCAGCGTCAGATG TGTAT
Ad1.17	AATGATACGGCGACCACCGAGATCTACACTTCGTGCATCGTCGGCAGCGTCAGATG TGTAT
Ad1.18	AATGATACGGCGACCACCGAGATCTACACAGGTTGCCTCGTCGGCAGCGTCAGATG TGTAT
Ad1.19	AATGATACGGCGACCACCGAGATCTACACCCTTATGTTCGTCGGCAGCGTCAGATG TGTAT

Ad1.20	AATGATACGGCGACCACCGAGATCTACACCAGCAACGTCGTCGGCAGCGTCAGATGTGTAT
Ad1.21	AATGATACGGCGACCACCGAGATCTACACGGTTCAATTCGTCGGCAGCGTCAGATGTGTAT
Ad1.22	AATGATACGGCGACCACCGAGATCTACACACATTCGTTCGTCGGCAGCGTCAGATGTGTAT
Ad1.23	AATGATACGGCGACCACCGAGATCTACACGATTCCCATCGTCGGCAGCGTCAGATGTGTAT
Ad1.24	AATGATACGGCGACCACCGAGATCTACACCGGACTGCTCGTCGGCAGCGTCAGATGTGTAT
Ad1.25	AATGATACGGCGACCACCGAGATCTACACAGCCGTTCTCGTCGGCAGCGTCAGATGTGTAT
Ad1.26	AATGATACGGCGACCACCGAGATCTACACATTGGGTCTCGTCGGCAGCGTCAGATGTGTAT
Ad1.27	AATGATACGGCGACCACCGAGATCTACACTGCATACTTCGTCGGCAGCGTCAGATGTGTAT
Ad1.28	AATGATACGGCGACCACCGAGATCTACACGGGCTTGGTCGTCGGCAGCGTCAGATGTGTAT
Ad1.29	AATGATACGGCGACCACCGAGATCTACACGACGTGGCTCGTCGGCAGCGTCAGATGTGTAT
Ad1.30	AATGATACGGCGACCACCGAGATCTACACGCAAATTTTCGTCGGCAGCGTCAGATGTGTAT
Ad1.31	AATGATACGGCGACCACCGAGATCTACACGCAGCCTCTCGTCGGCAGCGTCAGATGTGTAT
Ad1.32	AATGATACGGCGACCACCGAGATCTACACTCCGAGTTTCGTCGGCAGCGTCAGATGTGTAT
Ad1.33	AATGATACGGCGACCACCGAGATCTACACGCATTAAGTCGTCGGCAGCGTCAGATGTGTAT
Ad1.34	AATGATACGGCGACCACCGAGATCTACACACGATAACTCGTCGGCAGCGTCAGATGTGTAT
Ad1.35	AATGATACGGCGACCACCGAGATCTACACCCTGCGGGTCGTCGGCAGCGTCAGATGTGTAT
Ad1.36	AATGATACGGCGACCACCGAGATCTACACTGATTGTTTCGTCGGCAGCGTCAGATGTGTAT
Ad1.37	AATGATACGGCGACCACCGAGATCTACACGGCACGGATCGTCGGCAGCGTCAGATGTGTAT
Ad1.38	AATGATACGGCGACCACCGAGATCTACACGATCATTCTCGTCGGCAGCGTCAGATGTGTAT
Ad1.39	AATGATACGGCGACCACCGAGATCTACACATGGTCATTCGTCGGCAGCGTCAGATGTGTAT
Ad1.40	AATGATACGGCGACCACCGAGATCTACACCGTACCAATCGTCGGCAGCGTCAGATGTGTAT
Ad1.41	AATGATACGGCGACCACCGAGATCTACACCCAGTTTATCGTCGGCAGCGTCAGATGTGTAT
Ad1.42	AATGATACGGCGACCACCGAGATCTACACACCGGCCCTCGTCGGCAGCGTCAGATGTGTAT

Ad1.43	AATGATACGGCGACCACCGAGATCTACACCTAGAAGTTCGTCGGCAGCGTCAGATG TGTAT
Ad1.44	AATGATACGGCGACCACCGAGATCTACACCGCCAGATTCGTCGGCAGCGTCAGATG TGTAT
Ad1.45	AATGATACGGCGACCACCGAGATCTACACTCACATGGTCGTCGGCAGCGTCAGATG TGTAT
Ad1.46	AATGATACGGCGACCACCGAGATCTACACGAACTCGATCGTCGGCAGCGTCAGATG TGTAT
Ad1.47	AATGATACGGCGACCACCGAGATCTACACCCACCGTTTCGTCGGCAGCGTCAGATG TGTAT
Ad1.48	AATGATACGGCGACCACCGAGATCTACACTAAGTTACTCGTCGGCAGCGTCAGATG TGTAT
Ad1.49	AATGATACGGCGACCACCGAGATCTACACGAGACGTGTCGTCGGCAGCGTCAGAT GTGTAT
Ad1.50	AATGATACGGCGACCACCGAGATCTACACTTGCCTAATCGTCGGCAGCGTCAGATG TGTAT
Ad1.51	AATGATACGGCGACCACCGAGATCTACACTTAACTTGTCGTCGGCAGCGTCAGATG TGTAT
Ad1.52	AATGATACGGCGACCACCGAGATCTACACCTTTAACATCGTCGGCAGCGTCAGATG TGTAT
Ad1.53	AATGATACGGCGACCACCGAGATCTACACCGTAGACCTCGTCGGCAGCGTCAGATG TGTAT
Ad1.54	AATGATACGGCGACCACCGAGATCTACACTATTTGCGTCGTCGGCAGCGTCAGATG TGTAT
Ad1.55	AATGATACGGCGACCACCGAGATCTACACATCCAGGATCGTCGGCAGCGTCAGATG TGTAT
Ad1.56	AATGATACGGCGACCACCGAGATCTACACTGTTCCTGTCGTCGGCAGCGTCAGATG TGTAT
Ad1.57	AATGATACGGCGACCACCGAGATCTACACACGCGCAGTCGTCGGCAGCGTCAGAT GTGTAT
Ad1.58	AATGATACGGCGACCACCGAGATCTACACTCTGGCGATCGTCGGCAGCGTCAGATG TGTAT
Ad1.59	AATGATACGGCGACCACCGAGATCTACACAATCTACATCGTCGGCAGCGTCAGATG TGTAT
Ad1.60	AATGATACGGCGACCACCGAGATCTACACTACTGACCTCGTCGGCAGCGTCAGATG TGTAT
Ad1.61	AATGATACGGCGACCACCGAGATCTACACCGATAGGGTCGTCGGCAGCGTCAGAT GTGTAT
Ad1.62	AATGATACGGCGACCACCGAGATCTACACACTTAGAATCGTCGGCAGCGTCAGATG TGTAT
Ad1.63	AATGATACGGCGACCACCGAGATCTACACAGAGATCTTCGTCGGCAGCGTCAGATG TGTAT
Ad1.64	AATGATACGGCGACCACCGAGATCTACACGGTGAAGGTCGTCGGCAGCGTCAGAT GTGTAT
Ad1.65	AATGATACGGCGACCACCGAGATCTACACATCGAATGTCGTCGGCAGCGTCAGATG TGTAT

Ad1.66	AATGATACGGCGACCACCGAGATCTACACTCAAGAGCTCGTCGGCAGCGTCAGATG TGTAT
Ad1.67	AATGATACGGCGACCACCGAGATCTACACGCCCACGTTCGTCGGCAGCGTCAGATG TGTAT
Ad1.68	AATGATACGGCGACCACCGAGATCTACACTGGGCGGTTCGTCGGCAGCGTCAGAT GTGTAT
Ad1.69	AATGATACGGCGACCACCGAGATCTACACCCCTTGGATCGTCGGCAGCGTCAGATG TGTAT
Ad1.70	AATGATACGGCGACCACCGAGATCTACACATTACCGTTCGTCGGCAGCGTCAGATG TGTAT
Ad1.71	AATGATACGGCGACCACCGAGATCTACACAGTCCGAGTCGTCGGCAGCGTCAGAT GTGTAT
Ad1.72	AATGATACGGCGACCACCGAGATCTACACACTTGTTGTCGTCGGCAGCGTCAGATG TGTAT
Ad1.73	AATGATACGGCGACCACCGAGATCTACACGTAATACATCGTCGGCAGCGTCAGATG TGTAT
Ad1.74	AATGATACGGCGACCACCGAGATCTACACGGCGTCTATCGTCGGCAGCGTCAGATG TGTAT
Ad1.75	AATGATACGGCGACCACCGAGATCTACACGCGCTGCTTCGTCGGCAGCGTCAGATG TGTAT
Ad1.76	AATGATACGGCGACCACCGAGATCTACACGTGCCATTTCGTCGGCAGCGTCAGATG TGTAT
Ad1.77	AATGATACGGCGACCACCGAGATCTACACTAGGTATGTCGTCGGCAGCGTCAGATG TGTAT
Ad1.78	AATGATACGGCGACCACCGAGATCTACACAACACCTATCGTCGGCAGCGTCAGATG TGTAT
Ad1.79	AATGATACGGCGACCACCGAGATCTACACCTCCGAACTCGTCGGCAGCGTCAGATG TGTAT
Ad1.80	AATGATACGGCGACCACCGAGATCTACACCAACGGCATCGTCGGCAGCGTCAGAT GTGTAT
Ad1.81	AATGATACGGCGACCACCGAGATCTACACCAATGTAGTCGTCGGCAGCGTCAGATG TGTAT
Ad1.82	AATGATACGGCGACCACCGAGATCTACACGGCTACCCTCGTCGGCAGCGTCAGATG TGTAT
Ad1.83	AATGATACGGCGACCACCGAGATCTACACAAAGTCCGTCGTCGGCAGCGTCAGATG TGTAT
Ad1.84	AATGATACGGCGACCACCGAGATCTACACTTCCGCGGTCGTCGGCAGCGTCAGATG TGTAT
Ad1.85	AATGATACGGCGACCACCGAGATCTACACAGGCACTTTCGTCGGCAGCGTCAGATG TGTAT
Ad1.86	AATGATACGGCGACCACCGAGATCTACACCTTCAGTGTCGTCGGCAGCGTCAGATG TGTAT
Ad1.87	AATGATACGGCGACCACCGAGATCTACACGCCGGTAGTCGTCGGCAGCGTCAGAT GTGTAT
Ad1.88	AATGATACGGCGACCACCGAGATCTACACTTCAATCCTCGTCGGCAGCGTCAGATG TGTAT

Ad1.89	AATGATACGGCGACCACCGAGATCTACACCCACACACTCGTCGGCAGCGTCAGATG TGTAT
Ad1.90	AATGATACGGCGACCACCGAGATCTACACATATTATCTCGTCGGCAGCGTCAGATG TGTAT
Ad1.91	AATGATACGGCGACCACCGAGATCTACACCCGAAGCATCGTCGGCAGCGTCAGAT GTGTAT
Ad1.92	AATGATACGGCGACCACCGAGATCTACACGTATCGGTTCGTCGGCAGCGTCAGATG TGTAT
Custom Barcodes Adapter 2 (index i7):	
Adapter Name	Adapter Sequence
Ad2.1	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGGAGATGTG
Ad2.2	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGGAGATGTG
Ad2.3	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGGAGATGTG
Ad2.4	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGGAGATGTG
Ad2.5	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGGAGATGTG
Ad2.6	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGGAGATGTG
Ad2.7	CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTCTCGTGGGCTCGGAGATGTG
Ad2.8	CAAGCAGAAGACGGCATACGAGATCCTCTCTGGTCTCGTGGGCTCGGAGATGTG
Ad2.9	CAAGCAGAAGACGGCATACGAGATAGCGTAGCGTCTCGTGGGCTCGGAGATGTG
Ad2.10	CAAGCAGAAGACGGCATACGAGATCAGCCTCGGTCTCGTGGGCTCGGAGATGTG
Ad2.11	CAAGCAGAAGACGGCATACGAGATTGCCTCTTGTCTCGTGGGCTCGGAGATGTG
Ad2.12	CAAGCAGAAGACGGCATACGAGATTCCTCTACGTCTCGTGGGCTCGGAGATGTG
Ad2.13	CAAGCAGAAGACGGCATACGAGATCAGATCCAGTCTCGTGGGCTCGGAGATGTG
Ad2.14	CAAGCAGAAGACGGCATACGAGATACAAACGGGTCTCGTGGGCTCGGAGATGTG
Ad2.15	CAAGCAGAAGACGGCATACGAGATACCCAGCAGTCTCGTGGGCTCGGAGATGTG
Ad2.16	CAAGCAGAAGACGGCATACGAGATCCCAACCTGTCTCGTGGGCTCGGAGATGTG
Ad2.17	CAAGCAGAAGACGGCATACGAGATCACCACACGTCTCGTGGGCTCGGAGATGTG
Ad2.18	CAAGCAGAAGACGGCATACGAGATGAAACCCAGTCTCGTGGGCTCGGAGATGTG
Ad2.19	CAAGCAGAAGACGGCATACGAGATTGTGACCAGTCTCGTGGGCTCGGAGATGTG
Ad2.20	CAAGCAGAAGACGGCATACGAGATAGGGTCAAGTCTCGTGGGCTCGGAGATGTG
Ad2.21	CAAGCAGAAGACGGCATACGAGATTGTCCGCGGTCTCGTGGGCTCGGAGATGTG
Ad2.22	CAAGCAGAAGACGGCATACGAGATATATGGAAGTCTCGTGGGCTCGGAGATGTG
Ad2.23	CAAGCAGAAGACGGCATACGAGATAACGAATTGTCTCGTGGGCTCGGAGATGTG
Ad2.24	CAAGCAGAAGACGGCATACGAGATTCGACGCCGTCTCGTGGGCTCGGAGATGTG
Ad2.25	CAAGCAGAAGACGGCATACGAGATCACTTTGTGTCTCGTGGGCTCGGAGATGTG
Ad2.26	CAAGCAGAAGACGGCATACGAGATTTCAAGTAGTCTCGTGGGCTCGGAGATGTG
Ad2.27	CAAGCAGAAGACGGCATACGAGATGCTATCACGTCTCGTGGGCTCGGAGATGTG
Ad2.28	CAAGCAGAAGACGGCATACGAGATAATCTACTGTCTCGTGGGCTCGGAGATGTG
Ad2.29	CAAGCAGAAGACGGCATACGAGATCCGGCAATGTCTCGTGGGCTCGGAGATGTG
Ad2.30	CAAGCAGAAGACGGCATACGAGATCTTAGCAAGTCTCGTGGGCTCGGAGATGTG
Ad2.31	CAAGCAGAAGACGGCATACGAGATTAACTTATGTCTCGTGGGCTCGGAGATGTG
Ad2.32	CAAGCAGAAGACGGCATACGAGATCGAGTGATGTCTCGTGGGCTCGGAGATGTG
Ad2.33	CAAGCAGAAGACGGCATACGAGATCTGTTAACGTCTCGTGGGCTCGGAGATGTG
Ad2.34	CAAGCAGAAGACGGCATACGAGATCTACCATTGTCTCGTGGGCTCGGAGATGTG

Ad2.35	CAAGCAGAAGACGGCATACGAGATACGTGCTCGTCTCGTGGGCTCGGAGATGTG
Ad2.36	CAAGCAGAAGACGGCATACGAGATTGACGAAAGTCTCGTGGGCTCGGAGATGTG
Ad2.37	CAAGCAGAAGACGGCATACGAGATAATTCTTGGTCTCGTGGGCTCGGAGATGTG
Ad2.38	CAAGCAGAAGACGGCATACGAGATGGCATTTCGTCTCGTGGGCTCGGAGATGTG
Ad2.39	CAAGCAGAAGACGGCATACGAGATATGGCGTTGTCTCGTGGGCTCGGAGATGTG
Ad2.40	CAAGCAGAAGACGGCATACGAGATCTGCGAGGGTCTCGTGGGCTCGGAGATGTG
Ad2.41	CAAGCAGAAGACGGCATACGAGATGAGGTGTAGTCTCGTGGGCTCGGAGATGTG
Ad2.42	CAAGCAGAAGACGGCATACGAGATAAATGACCGTCTCGTGGGCTCGGAGATGTG
Ad2.43	CAAGCAGAAGACGGCATACGAGATTAAGATTGGTCTCGTGGGCTCGGAGATGTG
Ad2.44	CAAGCAGAAGACGGCATACGAGATAAGGCACAGTCTCGTGGGCTCGGAGATGTG
Ad2.45	CAAGCAGAAGACGGCATACGAGATTAATAAGAGTCTCGTGGGCTCGGAGATGTG
Ad2.46	CAAGCAGAAGACGGCATACGAGATACTAAGTCGTCTCGTGGGCTCGGAGATGTG
Ad2.47	CAAGCAGAAGACGGCATACGAGATGCTGGTCTGTCTCGTGGGCTCGGAGATGTG
Ad2.48	CAAGCAGAAGACGGCATACGAGATCTGTATTTGTCTCGTGGGCTCGGAGATGTG
Ad2.49	CAAGCAGAAGACGGCATACGAGATTTTCATAAGTCTCGTGGGCTCGGAGATGTG
Ad2.50	CAAGCAGAAGACGGCATACGAGATGACCCAAGGTCTCGTGGGCTCGGAGATGTG
Ad2.51	CAAGCAGAAGACGGCATACGAGATTTATTTGGGTCTCGTGGGCTCGGAGATGTG
Ad2.52	CAAGCAGAAGACGGCATACGAGATTTTAACGCGTCTCGTGGGCTCGGAGATGTG
Ad2.53	CAAGCAGAAGACGGCATACGAGATACAGGATGGTCTCGTGGGCTCGGAGATGTG
Ad2.54	CAAGCAGAAGACGGCATACGAGATCTTACTCCGTCTCGTGGGCTCGGAGATGTG
Ad2.55	CAAGCAGAAGACGGCATACGAGATGGAGCGTCGTCTCGTGGGCTCGGAGATGTG
Ad2.56	CAAGCAGAAGACGGCATACGAGATGCCGCGAAGTCTCGTGGGCTCGGAGATGTG
Ad2.57	CAAGCAGAAGACGGCATACGAGATGGGAACCGGTCTCGTGGGCTCGGAGATGTG
Ad2.58	CAAGCAGAAGACGGCATACGAGATTAGCCGGTGTCTCGTGGGCTCGGAGATGTG
Ad2.59	CAAGCAGAAGACGGCATACGAGATCCCATGAGGTCTCGTGGGCTCGGAGATGTG
Ad2.60	CAAGCAGAAGACGGCATACGAGATGCATTAAAGTCTCGTGGGCTCGGAGATGTG
Ad2.61	CAAGCAGAAGACGGCATACGAGATGACCGTTTGTCTCGTGGGCTCGGAGATGTG
Ad2.62	CAAGCAGAAGACGGCATACGAGATTTTGGATCGTCTCGTGGGCTCGGAGATGTG
Ad2.63	CAAGCAGAAGACGGCATACGAGATATCATCATGTCTCGTGGGCTCGGAGATGTG
Ad2.64	CAAGCAGAAGACGGCATACGAGATCGTGTTGGGTCTCGTGGGCTCGGAGATGTG
Ad2.65	CAAGCAGAAGACGGCATACGAGATTGTTGTTAGTCTCGTGGGCTCGGAGATGTG
Ad2.66	CAAGCAGAAGACGGCATACGAGATGGTTTACCGTCTCGTGGGCTCGGAGATGTG
Ad2.67	CAAGCAGAAGACGGCATACGAGATGGTCGATGGTCTCGTGGGCTCGGAGATGTG
Ad2.68	CAAGCAGAAGACGGCATACGAGATGTTCCCATGTCTCGTGGGCTCGGAGATGTG
Ad2.69	CAAGCAGAAGACGGCATACGAGATATTGGCCGGTCTCGTGGGCTCGGAGATGTG
Ad2.70	CAAGCAGAAGACGGCATACGAGATTCATTCCCGTCTCGTGGGCTCGGAGATGTG
Ad2.71	CAAGCAGAAGACGGCATACGAGATCCGAATACGTCTCGTGGGCTCGGAGATGTG
Ad2.72	CAAGCAGAAGACGGCATACGAGATATAGCTGAGTCTCGTGGGCTCGGAGATGTG
Ad2.73	CAAGCAGAAGACGGCATACGAGATAGATAAATGTCTCGTGGGCTCGGAGATGTG
Ad2.74	CAAGCAGAAGACGGCATACGAGATGCAACTGTGTCTCGTGGGCTCGGAGATGTG
Ad2.75	CAAGCAGAAGACGGCATACGAGATATCTCGGGGTCTCGTGGGCTCGGAGATGTG
Ad2.76	CAAGCAGAAGACGGCATACGAGATAGACATTAGTCTCGTGGGCTCGGAGATGTG
Ad2.77	CAAGCAGAAGACGGCATACGAGATGAATTGGCGTCTCGTGGGCTCGGAGATGTG
Ad2.78	CAAGCAGAAGACGGCATACGAGATGCACGGCGGTCTCGTGGGCTCGGAGATGTG
Ad2.79	CAAGCAGAAGACGGCATACGAGATTCGGTCAGGTCTCGTGGGCTCGGAGATGTG
Ad2.80	CAAGCAGAAGACGGCATACGAGATTCGAAATGGTCTCGTGGGCTCGGAGATGTG

Ad2.81	CAAGCAGAAGACGGCATACGAGATTGGCAAGCGTCTCGTGGGCTCGGAGATGTG
Ad2.82	CAAGCAGAAGACGGCATACGAGATTGGTAGAAGTCTCGTGGGCTCGGAGATGTG
Ad2.83	CAAGCAGAAGACGGCATACGAGATCGTCACGTGTCTCGTGGGCTCGGAGATGTG
Ad2.84	CAAGCAGAAGACGGCATACGAGATCGCGGACAGTCTCGTGGGCTCGGAGATGTG
Ad2.85	CAAGCAGAAGACGGCATACGAGATAAGTTTAAGTCTCGTGGGCTCGGAGATGTG
Ad2.86	CAAGCAGAAGACGGCATACGAGATGTTGTGGTGTCTCGTGGGCTCGGAGATGTG
Ad2.87	CAAGCAGAAGACGGCATACGAGATCCAGAGGCGTCTCGTGGGCTCGGAGATGTG
Ad2.88	CAAGCAGAAGACGGCATACGAGATGTGGGCGAGTCTCGTGGGCTCGGAGATGTG
Ad2.89	CAAGCAGAAGACGGCATACGAGATGCCTAGTGGTCTCGTGGGCTCGGAGATGTG
Ad2.90	CAAGCAGAAGACGGCATACGAGATGGCTTCGAGTCTCGTGGGCTCGGAGATGTG
Ad2.91	CAAGCAGAAGACGGCATACGAGATGTACATGCGTCTCGTGGGCTCGGAGATGTG
Ad2.92	CAAGCAGAAGACGGCATACGAGATACTCGAACGTCTCGTGGGCTCGGAGATGTG
Ad2.93	CAAGCAGAAGACGGCATACGAGATGCGCCCGGGTCTCGTGGGCTCGGAGATGTG
Ad2.94	CAAGCAGAAGACGGCATACGAGATTTAAATCTGTCTCGTGGGCTCGGAGATGTG
Ad2.95	CAAGCAGAAGACGGCATACGAGATCAATGGTGGTCTCGTGGGCTCGGAGATGTG
Ad2.96	CAAGCAGAAGACGGCATACGAGATGTCTTATTGTCTCGTGGGCTCGGAGATGTG