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# scNMT-seq v2

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ABSTRACT

scNMT-seq (single cell Nucleosome, Methylome, and Transcriptome sequencing) allows the parallel study of a single cell chromatin status, methylation profile, and transcriptome.

Here, we are developing and testing modifications of the scNMT-seq pipeline. The protocol is carried out in 96w plates and typically takes 4-5 days to complete.
The number of pre-amplification cycles is adjusted to tackle the problem of poor recovery after BS conversion.Primers are optimized for first-strand and second-strand synthesis to solve the problem of unmapped reads and poor amplification. Both are testified as compatible with theoriginal the original scNMTseq.

PROTOCOL CITATION

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KEYWORDS

Single-cell, scRNA-seq, G&T-seq, BS-seq, chromatin accessibility, DNA methylation, gene expression, rna, single cell, single, cell, ASAPCRN, scNMTseq, open chromatin



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MATERIALS TEXT

GpC Methyltransferase (M.CviPI) - 1,000 units **New England Biolabs Catalog #M0227L**

IGEPAL-CA630 **Sigma Aldrich Catalog #I3021 SIGMA-ALDRICH**

RNase Inhibitor **Thermo Fisher Catalog #N8080119**

RLT Plus Buffer **Qiagen**

Dynabeads MyOne Streptavidin T1 **Thermo Fisher Scientific Catalog #65601**

Superscript II **Invitrogen - Thermo Fisher Catalog #18064014**

ERCC RNA Spike-In Mix or order custom-made synthetic sequences **Thermo Fisher Scientific Catalog #4456740**

dNTP Mix (10 mM ea) **Thermo Fisher Catalog #18427013**

Nextera XT DNA Sample Preparation Kit, 96 samples **illumina Catalog #FC-131-1096**

Nextera XT Index Kit v2 Set A (96 indexes 384 samples) **illumina Catalog #FC-131-2001**

Betaine 5M **Sigma Aldrich Catalog #B0300**

Magnesium Chloride (MgCl2) Solution - 6.0 ml **New England Biolabs Catalog #B9021S**

DTT **Sigma Aldrich Catalog #D0632**

Kapa HiFi Hotstart ReadyMix (2x) **Kapa Biosystems Catalog #KK2612**

Ampure XP beads **Beckman Coulter Catalog #A63881**

Lambda DNA **Thermo Fisher Catalog #SD0011**

EZ-96 DNA Methylation-Direct MagPrep **Zymo Research Catalog #D5044**

Klenow (3'→ 5' exo-) (High Concentration) **Enzymatics Catalog #P7010-HC-L**

QuantiFluor® dsDNA System **Promega Catalog #E2670**

| A | B |
|---|---|
| **Name** | **Sequence (5' to 3')** |
| Oligo-dT primer | Biotin-TEG-AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN |
| TSO | AAGCAGTGGTATCAACGCAGAGTACATrGrG+G |
| IS PCR | AAGCAGTGGTATCAACGCAGAGT |
| Pre-amplification primer | Biotin-TGACTGGAGTTCAGACGTGTGCTCTTCCGATCT**HHHHHHH*H** |
| Adapter2 primer | ACACTCTTTCCCTACACGACGCTCTTCCGATCT**DDDDDDD*D** |
| PE 1.0 | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC*T |
| iPCRTag primer | CAAGCAGAAGACGGCATACGAGATXXXXXXXXGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T |

All oligos should be ordered with HPLC purification

## Cell isolation and GpC methylation

1

Prepare plates containing **2.5µl** GpC methylase reaction mixture in each well:

| A | B | C | D |
|---|---|---|---|
| **Component (initial)** | **Component(final)** | **Volume (µl)** | **Mastermix (110 samples)** |
| M.CviPI reaction buffer (10x) | 1x | 0.25 | 27.5 |
| M.CviPI (4U/µl) | 2U | 0.5 | 55 |
| SAM (**320µM**) | 160µM | 1.25 | 137.5 |
| IGEPAL (10%) | 0.1% | 0.025 | 2.75 |
| RNasein (20U/µl) | 1U/µl | 0.125 | 13.75 |
| Nuclease-free water | | 0.35 | 38.5 |

2

Isolate cells manually or using FACS in **2.5 µL** of GpC methylase reaction buffer

3

After cell isolation, spin down plates at **≥1000g** for **≥10s** **4 °C**

15m

4

Incubate the samples at **37 °C** for **00:15:00**

5

Stop reaction by adding **5 µL RLT plus buffer**

6

Stoe the plates at **-80 °C** until processed

## Oligo-dT$_{30}$VN bead preparation 30m

7 2m

Add **55 µL** Dynabeads into a new Eppendorf tube. Place the tube on a magnet for **00:02:00** and discard supernatant

8 2m

Resuspend beads in **200 µL Dynabead solution A (0.1 Molarity (M) NaOH, 0.05 Molarity (M) NaCl)**. Place the tube on a magnet for **00:02:00** and discard supernatant

9

Repeat step 8 once

10 2m

Resuspend beads in **200 µL Dynabead solution B (0.1 Molarity (M) NaCl)**. Place on a magnet for **00:02:00** and discard supernatant

11 20m

Resuspend the beads in **55 µL** of **2x B&W (2 Molarity (M) NaCl, 10 millimolar (mM) Tris-HCl, 1 millimolar (mM) EDTA)** and **55 µL** Biotinylated Oligo-dT30VN (**100 micromolar (µM)**). Incubate **00:20:00** on a thermomixer while shaking at **2000rpm** at **Room temperature**

*In the meantime, prepare the **bead resuspension buffer***

| A | B |
|---|---|
| Superscript FS buffer (5x) | 220µl |
| Nuclease-free Water | 825µl |
| **RNase inhibitor (20U/µl)** | **55µl** |

*After adding RNase inhibitor, use beads within 30min*

*In the meantime, prepare **1x B&W** buffer by mixing **440 µL** Nuclease-free water with **440 µL** **2x B&W** buffer*

12 2m

Place beads on a magnet for **00:02:00** and discard supernatant

13 2m

Resuspend the beads in **200 µL** **1x B&W** buffer. Place beads on a magnet for **00:02:00** and discard supernatant

14

Repeat step 13 **three** more times

15 Resuspend the beads in the **bead resuspension buffer**

Physical separation of mRNA and gDNA

16 Thaw the 96-well plate containing the single cell lysates on ice

17 Add **1 µL ERCC spike-ins** at 1:1Million - 1:128Million dilution to each sample using a multi-dispensing pipette. Run the pulse centrifugation program to spin ERCCs down to the bottom

18 Take 4 tubes( **1069 µL** per tube ) of **G&T wash buffer( 50 millimolar (mM) Tris−HCl pH 8.3 , 75 millimolar (mM) KCl, 3 millimolar (mM) MgCl2, 0.5% Tween 20 Solution)** and add to each tube **137.5 µL DTT** and **25 µL RNaseIn**

19 Add **50 µL G&T-Seqwash buffer** per well to the"G&T-Seq wash plate"

20 Add **10 µL Oligo-dT beads** per well to the"bead plate"

21 Add an empty **non-skirted** 96 well plate labeled "gDNA collection"

22 Spin all plates and run the adapted G&T-separation program robotically or manually.

*While the separation program is running, prepare the **RT master mix***

| A | B | C | D |
|---|---|---|---|
| **Component (C_initial)** | **C_final** | **Volume(µl)** | **Mastermix (110 samples)** |
| dNTP (10mM) | 1mM | 0.5 | 55 |
| TSO (100µM) | 1µM | 0.05 | 5.5 |
| MgCl2 (1M) | 6mM | 0.03 | 3.3 |
| Betain (5M) | 1M | 1 | 110 |
| S II First strand buffer (5x) | 1x | 1 | 110 |
| DTT (100mM) | 5mM | 0.25 | 27.5 |
| Nuclease-free water | | 1.8 | 198 |
| **RNase inh (20U/µl)** | **0.5U/µl** | **0.125** | **13.75** |
| **Superscript reverse transcriptase II (200U/µl)** | **10U/µl** | **0.25** | **27.5** |

*Adding enzyme within less than 30 min before running the Reverse Transcription program*

Separation is performed robotically on the Hamilton platform in this protocol. If performed manually, steps should be as follows

22.1

Manually pipette **10 µL** of prepared **oligo-dT beads** to each well of the **sample plate** using a multichannel pipette

20m

22.2

Mix at maximum speed for **00:20:00**

5m

22.3

Place on magnet for **00:05:00** . Aspirate **17.5 µL** and transfer to the empty low-bind plate for gDNA collection

22.4

Add **15 µL** of G&T-seq wash buffer off magnet.

10m

22.5

Mix at maximum speed for **00:10:00**

2m

22.6

Place on magnet for **00:02:00** . Aspirate **15 µL** and transfer to the empty low-bind plate for gDNA collection

22.7 Repeat steps 22.3-22.6 twice more

Lysate (17.5ul) combined with 3 washes (15ul each) should now have been collected into the gDNA plate

## Reverse transcription 1h 45m

23 Collect the polyA(+) mRNA plate and using the multi-dispenser dispense **5 µL RT master mix** into each well of the bead-containing 96-well plate

24 Seal the mRNA and gDNA plates and spin.

Store gDNA at **-80 °C** until processed

1h 45m

25 Incubate the polyA(+) mRNA 96-well plate on a **thermomixer C** using the program below (approx. duration **01:45:00** )

| A | B | C | D |
|---|---|---|---|
| **Cycle** | **Temp (°C)** | **Time** | **Mixing (rpm)** |
| 1 | 42 | 2 min | 2000 |
| 2 | 42 | 60 min | 1500 |
| 3 | 50 | 30 min | 1500 |
| 4 | 60 | 10 min | 1500 |

26 *In the meantime prepare **PCR mastermix***

| A | B | C |
|---|---|---|
| **Component** | **Volume(µl)** | **Mastermix (110 samples)** |
| KAPA HiFi HotStart ReadyMix (2x) | 6.25 | 687.5 |
| IS PCR primer (10µM) | 0.124 | 13.64 |
| Nuclease-free water | 1.13 | 124.3 |

## PCR amplification of cDNA 30s

27 Add **7.5 µL PCR reaction mastermix**, seal the plate and centrifuge

30s

28 Resuspend the beads for **00:00:30** at **2000rpm** using the Thermomixer

29 Perform cDNA amplification as follows

| A | B | C |
|---|---|---|
| **Cycles** | **Temperature(°C)** | **Time** |
| 1 | 98 | 3 min |
| 18-25 | 98 | 20 s |
| | 67 | 15 s |
| | 72 | 6 min |
| 1 | 72 | 5 min |
| 1 | 4 | Hold |

Amplification cycles differ

## PCR cleanup of amplified cDNA 22m 10s

30 Add **12.5 µL** **Agencourt AMPure beads** (1:1 ratio), mix thoroughly by pipetting up and down

5m

31 Incubate **00:05:00** at **Room temperature**

5m

32 Pellet the beads on a Low-elution magnet for **00:05:00**

33 Remove the supernatant without disturbing the beads

10s

34 Wash the beads twice with **150 µL** of freshly prepared 80% ethanol for **00:00:10**

7m

35 Allow the beads to dry for approximately **00:05:00** . Resuspend in **25 µL** nuclease-free water. Incubate for **00:02:00** **Room temperature**

5m

36 Return the 96-well plate to the magnet and allow the Agencourt AMPure beads to settle for **00:05:00**

37 Carefully transfer the supernatant to a new 96-well plate

Quality control: QUBIT+BIOANALYZER
*expected cDNA concentration: >= 1ng/µl*
*expected cDNA length: 500-2000bp, peaking at 1-1.5kb*

## Library preparation of cDNA (Nextera XT) 9m

38 Dilute the **cDNA** of each sample to **0.2ng/µl** with nuclease-free water

39 Add **2.5 µL** **Tagment DNA(TD) buffer** to a new Hard-Shell skirted 96-well plate

40 Add **1.25 µL** **diluted cDNA** and **1.25 µL** **amplicon tagment mix** (ATM) to TD buffer and mix

1m

41 Centrifuge the plate at **280 x g, 20°C, 00:01:00**

42 Incubate on a thermal cycler

| A | B | C |
|---|---|---|
| **Segment** | **Temp(°C)** | **Duration(min)** |
| 1 | 55 | 10 |
| 2 | 10 | Hold |

43 Add **1.25 µL** Neutralize Tagment Buffer (NT)

1m

44 Vortex & spin down at **800 x g, 20°C, 00:01:00**

5m

45 Incubate at **Room temperature** **00:05:00**

46 Add **1.25 µL** **Index (i7) adapter** to each column and **1.25 µL** **Index 2 (i5) adapter** to each row

47

Add **3.75 µL** **Nextera PCR mastermix** and mix

48 1m

Centrifuge the samples at **280 x g, 20°C, 00:01:00** and amplify as follows:

| A | B | C |
|---|---|---|
| **Cycle** | **Temp (°C)** | **Duration** |
| 1 | 72 | 3min |
| 2 | 95 | 30s |
| 3-14 | 95 | 10s |
| | 55 | 30s |
| | 72 | 30s |
| 15 | 72 | 5min |
| 16 | 4 | Hold |

49 1m

Centrifuge the plate at **280 x g, 20°C, 00:01:00**

50

Purify libraries at a **0.66:1 ratio** and elute in **12.5 µL** **EB buffer**

*Libraries can be stored for at least a year at -20°C*

51

Pool libraries and quantify using qPCR

*expected pool concentration: 4nM*
*expected pool size: 250-1500bp*

scBS-seq library preparation (gDNA)

52

Bisulfite conversion 39m

53 19m

Prepare the CT conversion reagent by mixing **7.9 mL** **M-Solubilisation buffer** and **3 mL** **M-Dilution buffer** and **00:15:00** vortexing at **Room temperature**
Finally,add **1.6 mL** **M-Reaction buffer** and vortex **00:04:00** at **Room temperature**

54 Add **32.5 µL** **AMPure XP beads** to the gDNA plate (0.65:1 ratio)

55 Incubate **00:20:00** **Room temperature** 20m

56 Place the plate on the magnet for **00:20:00** and discard the supernatant 20m

57 Wash the beads twice with **200 µL** 80% ethanol

58 Resuspend the beads in **10 µL** elution buffer, optionally containing 60fg unmethylated lambda DNA

> Do not transfer the samples from the beads
> Do not dry the beads after the second wash, a dry step when purifying gDNA lowers recovery

59 Add **65 µL** CT conversion reagent **without mixing**

> Watch out for bubbles, centrifuge shortly if necessary

60 Incubate the mixture as follows:

| A | B | C |
|---|---|---|
| Segment | Temperature(°C) | Duration(min) |
| 1 | 98 | 8 |
| 2 | 65 | 180 |
| 3 | 4 | Hold |

*BS converted DNA is stable for 3 days at -20°C or 20h at 4°C*

## Purification of the bisulfite converted DNA 33m

61 Mix **300 µL** M-binding buffer and **5 µL** MagBinding beads

*Tip: to minimize loss of sample due to pipetting use a thermomixer to mix instead of pipetting*
*Use a deep-well plate*

62 5m

Transfer the samples to the M-binding buffer - MagBinding beads mix and incubate **00:05:00**
**Room temperature**

63 3m

Pellet the beads on a magnet for **00:03:00** and discard the supernatant

64 Resuspend the beads in **200 µL** M-Wash buffer

65 15m

Pellet beads on the magnet and discard the supernatant. Resuspend the beads in **100 µL** M-Desulphonation buffer and incubate **00:15:00** **Room temperature**

*The beads sink quite fast to the bottom, during these 15 mins you can slowly mix on regular basis with the thermomixer*

66

Pellet beads on the magnet and discard the supernatant. Wash the beads twice with **200 µL** M-Wash buffer

67 10m

Dry the beads on a heating element at **55 °C** for **00:10:00**

*In the meantime, prepare the **pre-amplification mix** as follows*

| A | B | C | D |
|---|---|---|---|
| **Component** | **Amount (µl)** | **Final concentration** | **Mastermix (110 samples)** |
| Blue buffer (10×) | 4 | 1x | 440 |
| dNTP mix (10mM) | 1.6 | 0.4mM | 176 |
| Preamp Oligo (**10 µM**) | 1.6 | 0.4 µM | 176 |
| H2O | 32.8 | | 3608 |
| Total volume | 40 | | 4400 |

Pre-amplification 8m 5s

68

Resuspend the beads in a **40 µL pre-amplification mix**

69 4m

Incubate the mixture at **55 °C** for **00:04:00** and place it on the magnet

70

After the beads are pelleted transfer **39 µL** to a new plate

71 3m 10s

Incubate the samples **00:03:00** at **65 °C** and immediately cool on a pre-cooled aluminum rack

Centrifuge the plate at **500 x g, Room temperature, 00:00:10**

72

Add **1 µL** **klenow exo- polymerase** (50U/µl)

Vortex the samples and amplify as follows:

| A | B | C | D |
|---|---|---|---|
| **Segment** | **Temp (°C)** | **Duration (min)** | **Ramp speed (°C/min)** |
| 1 | 4 | 5 | - |
| 2 | 4-37 | 8.25 | 4 |
| 3 | 37 | 30 | - |
| 4 | 4 | Hold | |

73 *In the meantime, prepare 6 tubes of **pre-amplification mix***

Only add klenow exo to the mix before use

| A | B | C | D |
|---|---|---|---|
| **Component** | **Amount (µl)** | **Final concentration** | **Mastermix ( samples)** |
| Blue buffer (10×) | 0.25 | 1x | |
| dNTP mix (10mM) | 0.1 | 0.4mM | |
| Preamp Oligo (**10 µM**) | 1 | 4 µM | |
| **Klenow exo- (50 U/µl)** | 0.5 | 10 U/µl | |
| H2O | 0.65 | | |
| Total volume | 2.5 | | |

45s

74 Heat the plate to **95 °C** for **00:00:45** and transfer it to an aluminum rack pre-cooled on ice

10s

75 Centrifuge the plate at 500g for **00:00:10** at 15-25°C

76 Add **2.5 µL** of the **pre-amplification mix**

77 Repeat steps 72-76 **five more times**

78 Incubate as follows:

| A | B | C | D |
|---|---|---|---|
| **Segment** | **Temp (°C)** | **Duration (min)** | **Ramp speed (°C/min)** |
| 1 | 4 | 5 | - |
| 2 | 4-37 | 8.25 | 4 |
| 3 | 37 | 90 | - |
| 4 | 4 | Hold | |

*The first-strand product can be stored ON at 4°C or for at least a month at -20°C*

## Exonuclease I treatment 1h

79 Dilute the samples to a volume of **98 µL** with **nuclease-free water**

80 1h

Add **2 µL** **exonuclease I** (20U/µl) to the pre-amplified product and incubate **01:00:00** at **37 °C** with the **heated lid set to** **50 °C**

## Purification 18m

81 Add **75 µL** **AMPure XP beads** (0.75:1 ratio) and mix thoroughly by pipetting up and down

*Tip check the volume of some samples first and adjust volumes of beads to add accordingly*

82 10m

Incubate **00:10:00** **Room temperature**
*In the meantime, prepare **Adaptor 2 mix***

| A | B | C | D |
|---|---|---|---|
| **Component** | **Amount (µl)** | **Final concentration** | **Mastermix ( samples)** |
| Blue buffer (10×) | 4.7 | 1x | |
| dNTP mix (10mM) | 1.9 | 0.4mM | |
| Adapter 2 Oligo **(10 µM)** | 1.9 | 0.4µM | |
| H2O | 38 | | |
| Total volume | 46.5 | | |

83 3m

Place on the magnet for **00:03:00** and discard the supernatant

84 Add **200 µL** of 80% (vol/vol) ethanol while keeping the plate on the magnet then discard ethanol after ±10sec

85 5m

Repeat 84 once. Dry the AMPure XP beads for **00:05:00** **Room temperature**

Adapter 2 tagging 12m

86 Resuspend the beads in **46.5 µL** **Adapter 2 mix**

87 10m
Incubate for **00:10:00** **Room temperature**

88 Transfer samples to a new plate

89 45s
Heat mixture to **95 °C** for **00:00:45** then immediately cool on ice using an aluminum rack

90 10s
Spin down at 500g for **00:00:10** at 15–25°C

91 10s
Add **1 µL** **Klenow exo-** (50 U/µl), vortex gently, and spin down at 500g for **00:00:10** at 15–25°C

92 incubate as follows:

| A | B | C | D |
|---|---|---|---|
| **Segment** | **Temp (°C)** | **Duration (min)** | **Ramp speed (°C/min)** |
| 1 | 4 | 5 | - |
| 2 | 4-37 | 8.25 | 4 |
| 3 | 37 | 30 | - |
| 4 | 4 | Hold | |

93 *In the meantime, prepare 1 tube of* ***Adapter 2 mix***

| A | B | C | D |
|---|---|---|---|
| **Component** | **Amount (µl)** | **Final concentration** | **Mastermix ( samples)** |
| Blue buffer (10×) | 0.25 | 1x | |
| dNTP mix (10mM) | 0.1 | 0.4mM | |
| Preamp Oligo (**10 µM**) | 1 | 4 µM | |
| Klenow exo- (50 U/µl) | 0.5 | 10 U/µl | |
| H2O | 0.65 | | |
| Total volume | 2.5 | | |

94 45s

Heat the plate to **95 °C** for **00:00:45** and transfer it to an aluminum rack pre-cooled on ice

95 10s

Centrifuge the plate at 500g for **00:00:10** at 15-25°C

96

Add **2.5 µL** of the **Adapter 2 mix**

97

Incubate as follows:

| A | B | C | D |
|---|---|---|---|
| **Segment** | **Temp (°C)** | **Duration (min)** | **Ramp speed (°C/min)** |
| 1 | 4 | 5 | - |
| 2 | 4-37 | 8.25 | 4 |
| 3 | 37 | 90 | - |
| 4 | 4 | Hold | |

Purification 21m 10s

98

Add **37.5 µL** AMPure XP beads (**0.75:1 ratio**)

99 10m

Incubate **00:10:00** at room temperature

*In the meantime, prepare the **library amplification mix***

| A | B | C | D |
|---|---|---|---|
| **Component** | **Amount (µl)** | **Final Concentration** | **Mastermix ( samples)** |
| KAPA HIFI HotStart ReadyMix (2x) | 25 | 1x | |
| PE1.0 **(10µM)** | 1 | 0.2µM | |
| Nuclease-free water | 23 | | |
| Total volume | 49 | | |

100 3m

Place on a magnet for **00:03:00**

101 3m

Place on a magnet for **00:03:00** and discard the supernatant

102 10s

Add **200 µL** ethanol (70%) without disturbing the beads. After **00:00:10** remove ethanol

103 5m

Repeat step 102 once then dry beads **00:05:00** at room temperature

## Library amplification 10m

104

Resuspend the beads in **49 µL** **library amplification mix**

105 10m

Incubate the mixture **00:10:00** **Room temperature**

106

Place on a magnet and transfer supernatant to a new plate

107

**Add** **1 µL** **10µM reverse iPCRTag primer**(containing a sample-specific index)

Amplify as follows:

| A | B | C |
|---|---|---|
| **Cycles** | **Temperature (°C)** | **Time** |
| 1 | 95 | 3 min |
| 17-20 | 98 | 80 s |
| | 65 | 30 s |
| | 72 | 30 s |
| 1 | 72 | 3 min |
| 1 | 4 | Hold |

*The PCR product can be stored ON at 4°C or for at least a month at -20°C*

## Purification of amplified libraries 28m

108

**Add 37.5µl AMPure XP beads** (0.75:1 ratio) and mix well

109 10m

Incubate **00:10:00** **Room temperature**

110 3m

Place on the magnet for **00:03:00** and discard supernatant

111

Add **200 µL** ethanol (70%) without removing the plate from the magnet then discard the ethanol

112 5m

Repeat step 111 once then dry beads **00:05:00**

113

Resuspend the beads in **15 µL** **EB**

114 10m

Incubate **00:10:00** **Room temperature**

115

Place on a magnet then transfer supernatant to a new plate

Library quantity and quality can be checked using Qubit HS Assay and Bioanalyzer
*expected gDNA concentration: >= 1ng/µl*
*expected fragment length: >200bp and on average 400-600bp*

*Libraries can be stored for at least a year at -20°C*