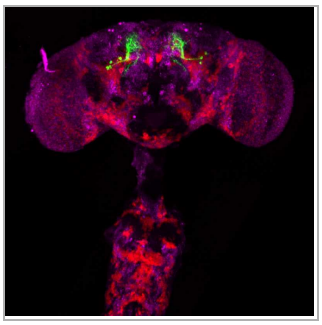

DEC 11, 2022

WORKS FOR ME 2

Expansion-assisted iterative fluorescence in situ hybridization (EASI-FISH) in Drosophila CNS

COMMENTS 2

DOI
dx.doi.org/10.17504/protocols.io.5jyl8jmw7g2w/v1

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ABSTRACT

A straightforward, robust, and reliable protocol (EASI-FISH) that utilizes expansion microscopy and the hybridization chain reaction for multiplexed *in situ* hybridization in thick slices of the mouse brain has recently been described (Wang et al., 2021). Below details a modified version of the EASI-FISH protocol for adult *Drosophila* CNS, which includes antibody detection of fluorescent reporters. The protocol also works well for larval CNS and is expected to be applicable to other tissue types.

ATTACHMENTS

Fly_EASI_FISH_published_protocol.docx
Materials_for_EASI_FISH.xlsx
LIGHTSHEETHOLDER_V18R_and_V20M_DOCUMENTATION.zip

DOI

dx.doi.org/10.17504/protocols.io.5jyl8jmw7g2w/v1

PROTOCOL CITATION

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Drosophila CNS, In situ hybridization, HCR, Expansion Microscopy, EASI-FISH

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Reference:

Wang et al., EASI-FISH for thick tissue defines lateral hypothalamus spatio-molecular organization. ***Cell***184 (2021) 6361-6377.e24.

https://pubmed.ncbi.nlm.nih.gov/34875226/

MATERIALS TEXT

Solutions for Fly brain dissection

- 2% PARAFORMALDEHYDE 16% Aqueous SOL. EM GRADE **Electron Microscopy Sciences Catalog #15710** in S2 medium.

- Schneider's Drosophila Medium **Thermo Fisher Catalog #21720024**
- PBT (0.5% Triton).

- 70% Ethanol (in nuclease-free water).

Solutions for Day 1: Labelling RNA

- MOPS (Fine White Crystals/Molecular Biology) **Fisher Scientific Catalog #bp308100**. Store Room temperature.

- Melphalan-X (Stock [M] 2 mg/mL; working [M] 1 mg/mL). Store -20 °C.

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- Acryloyl-X, SE **Thermo Fisher Scientific Catalog #A20770**, Stock [M] 10 mg/mL; working [M] 0.1 mg/mL. Store -20 °C.
- Press-to-Seal™ Silicone Isolator with Adhesive, eight wells, 9 mm diameter, 0.5 mm deep **Thermo Fisher Catalog #P24743**

- Poly-L-Lysine solution 10ml **TEDPELLA Catalog #18026**, 1.6 mL + 3.2 µL PHOTO-FLO 200 SOLUTION **Electron Microscopy Sciences Catalog #74257**. Store 4 °C.

- PBS-Triton (0.1%)

- 0.2 ml PCR tubes (USA Scientific; 1402-4700).

- RNase Away (Thermo Scientific; 7003).

Solutions for Day 2: Gelation and Proteinase K digestion

- Stock-X. Store -20 °C.
- 10% Ammonium Persulfate **Sigma Catalog #A3678**. Store -20 °C.
- 10% NNN'N'-Tetramethylethylenediamine **Sigma Aldrich Catalog #T22500**. Store -20 °C.
- 0.5% 4-Hydroxy-TEMPO **Sigma Catalog #176141**). Store -20 °C.
- Proteinase K, Molecular Biology Grade - 2 ml **New England Biolabs Catalog #P8107S**, [M] 800 U/ml). Store -20 °C.
- [M] 50 millimolar (mM) Proteinase K Buffer: Store Room temperature.
- Small paint brush.

Solutions for Day 3: Hybridization

- Hybridization Buffer (Molecular Instruments, Store -20 °C).
- Probe Wash Buffer (Molecular Instruments, Store -20 °C).
- DNA oligo probes (Designed and made by Molecular Instruments, Store -20 °C).
- DAPI (Sigma D9534)/ PBS **Fisher Scientific Catalog #BP24384** at [M] 500 ng/ml.

Solutions for Day 5: Hybridization Chain Reaction (HCR)

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- Amplification Buffer (Molecular Instruments, Store 4 °C).
- Fluorescent Hairpins (448 or 546 from Molecular Instruments or 669 conjugated in lab, Store -20 °C).
- 5x SSCT (5x SSC, 0.1% Tween in Nuclease Free Water). Store Room temperature.
- 0.5x SSCT (0.5x SSC, 0.1% Tween in Nuclease Free Water). Store Room temperature.
- Invitrogen GFP Polyclonal Antibody Alexa Fluor™ 488 **Thermo Fisher Scientific Catalog #A-21311**.
- UltraPure™ BSA (50 mg/mL) **Thermo Fisher Scientific Catalog # AM2616**.

Solutions for Stripping Probes and Hairpins for Multiplexing

DNAse I, RNAse-free **Qiagen Catalog #79254**.

Note

DNAse1 buffer

A	B
Tris-HCL pH 8.0	10 mM
MgCl2	2.5 mM
CaCl2	0.5 mM

Recipes and Reagents

200mM MOPS Buffer (10X Stock):

1046.5 mg in 25 mL in NFW, pH to 7.7 with [M] 10 Normality (N) NaOH. Store -20 °C.

Melphalan stock (2.5mg/ml)

Melphalan **Cayman Chemical Company Catalog #16665**
DMSO, Anhydrous **Thermo Fisher Catalog #D12345**

- Dissolve 2.5 mg per ml in anhydrous DMSO (Invitrogen, D12345).
- Dissolve, heat to 37 °C and vortex vigorously and place on a shaker.
- May take an hour to dissolve.

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- Aliquot in 800 µL batches.
- Store in a desiccated environment at -20 °C.

Acryloyl-X (AcX) stock (10 mg/ml)

Acryloyl-X, SE **Thermo Fisher Scientific Catalog #A20770**

- Dissolve in anhydrous DMSO.
- Aliquot in 200 µL batches.
- Any extra aliquot in 5 µL batches (for extra AcX).
- Store in a desiccated environment at -20 °C.
- Don't re-use AcX after thawing.

Melphalan-X (2mg/ml):

- Combine an equal concentration of Acryloyl-X ([M] 10 mg/mL) and Melphalan ([M] 2.5 mg/mL) (1-part AcX to 4-parts Melphalan (ie. 200 µL : 800 µL).
- Incubate Overnight at Room temperature with shaking.
- Store in 50 µL aliquots in a desiccated environment at -20 °C.
- Use at [M] 1 mg/mL by 1:1 dilution with [M] 20 millimolar (mM) MOPS.

Stock X:

[M] 4.04 Molarity (M) Sodium Acrylate* – made from Acrylic Acid as it is made at variable purity
40% Acrylamide Solution **Bio-rad Laboratories Catalog #1610140**
2% Bis Solution **Bio-rad Laboratories Catalog #1610142**
NaCl (5 M) RNase-free **Thermo Fisher Scientific Catalog #AM9760G**
Nuclease-free water **Ambion Catalog #AM9932**
10X PBS (ThermoFisher; AM9625)

4.04M Sodium Acrylate stock solution

- In a fume hood, place 5.5 mL of acrylic acid into a 50mL tube. Place tube in a Room temperature water bath (e.g. a beaker).
- Add 4.5 mL water.
- Add 7.2 mL [M] 10 Molarity (M) NaOH gradually to prevent excessive heating and evaporation/boiling.
- Remove tube from hood (at this point most of the acrylic acid has been converted to non-volatile sodium acrylate).
- Add [M] 1 Molarity (M) NaOH (nominally 1 mL) gradually until the pH is between 7.5 and 8 using a pH meter, at

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Room temperature. Do not use pH test strips.

- Add water up to a final volume of 20 mL.

Note

For 9.4 mls Stock X (not including APS +TEMED + 4HT):

A	B
4.04M Sodium Acrylate	2275 ul
40% Acrylamide	625 ul
2% N,N MethylBisacrylamide	750 ul
5M NaCl	4000 ul
10x PBS	1000 ul
Nuclease Free Water	750 ul

10% APS:

A	B
APS	100 mg
H20	900 ul

10% Temed:

A	B
TEMED	100 ul
H20	900 ul

0.5% 4HT:

A	B
4HT	5 mg
H20	995 ul

Note

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50mM ProK/SDS Buffer (50ml)

EDTA (0.5 M, pH 8.0, nuclease-free) **Thermo Fisher Scientific Catalog #AM9260G**

10% SDS solution **Thermo Fisher Scientific Catalog #15553027**

A	B
Tris-HCL- pH8	50 mM
EDTA	1 mM
TritonX	0.5 %
NaCl	50 mM
SDS	0.3 %

A	B
1 M Tris> 50 mM	2.5 ml
10 % Triton> 0.5 %	2.5 ml
5 M NaCl> 50 mM	0.5 ml
0.5 M EDTA	100 ul
10% SDS > 0.3% SDS	1.5 ml
Nuclease Free Water	42.9 ml

Note

RNase-Free DNase1

- Add 550 µL DNAase1 Buffer to DNase1 powder. Mix.

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- Aliquot 50 µL per PCR tube, store @ -20 °C.

DNase1 buffer (50ml)

A	B
1 M Tris-HCL > 10 mM	500 ul
1 M MgCl2 > 2.5 mM	125 ul
1 M CaCl2 > 0.5 mM	25 ul
Nuclease Free Water	49.350 ml

HCR Wash Buffers (50 ml)

5x SSCT:

SSC, RNase-free, 20× **Ambion Catalog #AM9763**

A	B
20x RNase free-SSC (ThermoFisher, AM 976	12.5 ml
10% Tween	500 ul
Nuclease Free Water	37 ml

0.5x SSCT:

A	B
20x RNase free-SSC	1.25 ml
10% Tween	500 ul
Nuclease Free Water	48.25 ml

Reagents for JF-669 conjugation to unlabelled hairpins:

- Janelia Fluor® 669 **Tocris Catalog #6420** Store -20 °C.
- [M] 1 nanomolar (nM) unlabelled amine-modified hairpins (~70mer, Molecular Instruments, Store -20 °C).
- Acetonitrile anhydrous **Thermo Fisher Scientific Catalog #042311-K7**
- [M] 0.1 Molarity (M) Sodium Bicarbonate pH 8-9.
- DMSO, Anhydrous **Thermo Fisher Catalog #D12345**
- QIAquick Nucleotide Removal Kit (250) **Qiagen Catalog #28306**

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- SCREW CAP MICROCENTRIFUGE TUBES SELF-STANDING AMBER (0.5 ml) **USA Scientific Catalog #1405-9707**

Equipment

Equipment	
Savant™ SpeedVac™ SPD120 Vacuum Concentrator and Kits	NAME
Vacuum Concentrator	TYPE
Savant	BRAND
SPD120-230	SKU
https://www.thermofisher.com/order/catalog/product/SPD120-230	LINK

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ATTACHMENTS

Fly_EASI_FISH_published_protocol.docx

Materials_for_EASI_FISH.xlsx

LIGHTSHEET HOLDER_V18R_and_V20M_DOCUMENTATION.zip

Fly brain dissection

1 Dissect fly CNS in S2 medium.
(for more details see attached protocol)

55m

2 Fix up to 20 brains or 10 CNS in 2 mL of 2% PFA/S2 medium for 00:55:00 in the dark on a nutator.

3 Rinse sample 1 x 2 mL PBST (0.5% Triton).

15m

4 Wash sample 4 x 00:15:00 1 mL PBST(0.5%Triton) on a nutator.

5 Rinse sample 1 x 2 mL 70% EtOH.

6 Store brains in 2 mL 70% EtOH @ 4 °C for up to 6 months.

Day 1: Labelling RNA

7 Transfer brains to a 0.2ml PCR tube (2-4 brains per tube).

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8 Rehydrate with 2 x 5 min wash in 150 µL PBT (0.1%).

8.1 Rehydrate with 2 x 00:05:00 wash in 150 µL PBT (0.1%) (1/2). 5m

8.2 Rehydrate with 2 x 00:05:00 wash in 150 µL PBT (0.1%) (2/2). 5m

9 Incubate brains 1 x 00:30:00 in 150 µL [M] 20 millimolar (mM) MOPS Buffer. 30m

10 Thaw Melphalan-X (MelphX) and Acryloyl-X (Ac-X) solutions.

11 Using a P20 pipette, take off as much MOPS buffer from the brains as possible.

12 Dilute Melphalan-X stock 1:1 with MOPS Buffer (to 1mg/ml).

13 Add 1/100 Ac-X ([M] 10 mg/mL) to Melphalan-X working solution. Vortex, mix, and spin.

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14 Add 30 µL of Melphalan-X/AcX solution to each PCR tube and gently mix.

15 Incubate Overnight @ 37 °C. 2m

16 Prepare gel chambers for gelation the next day. Wipe a non-charged slide with RNase away. Adhere a gasket (4-6 wells max) and coat the glass surface of the chamber with 1 µL poly-Lysine using a P20 pipette tip. Air dry and repeat.

Chambers.jpg

Day 2: Gelation and Proteinase K digestion

17 Wash brains 2 x 2 min 150 µL PBT and 1 x 2 mins 150 µL PBS.

17.1 Wash brains 2 x 00:02:00 150 µL PBT and 1 x 00:02:00 150 µL PBS. 4m

17.2 Wash brains 2 x 00:02:00 150 µL PBT and 1 x 00:02:00 150 µL PBS. 4m

18 Thaw Stock-X and 4HT, Temed and APS. Vortex well and keep On ice.

19 Gently stick down brains in the center of the chamber, once stuck down, carefully add a drop of PBS to prevent dehydration.

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Note

20 Mix together Stock-X and 4HT, Temed and APS at a ratio of 94:2:2:2. Vortex.

Note

21 Remove PBS from chamber with pipette tip and carefully wick away remaining PBS with a tissue.

22 10m

Pipette 40 µL of gel solution, on top of the brain, to each chamber. Incubate slide in the fridge (4 °C) for 00:10:00 .

23 Take off gel solution and repeat step 22.

Note

24 10m

Take off the gel solution and gasket surface adhesive. Add a final 40 µL of gel solution and gently place a cover slip over the chamber. Gently press to seal the coverslip and incubate @ 4 °C for 00:10:00 .

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Note

3h 30m

25 Polymerize the gel @ 37 °C for 01:30:00 to 02:00:00 .

26 Cool gels on the bench for a few minutes.

27 Take off the chamber lid and gasket with a razor blade. Trim the gels into a rectangle and nick the top right-hand corner to track the orientation of the sample.

28 Take off the gel from the slide with a paintbrush that has been wetted with a small amount of ProK Buffer and transfer it to a 2 mL Eppendorf.

2h

29 Incubate each gel with 1 mL ProK Buffer and 10 µL (1/100) ProK Enzyme @ 37 °C Overnight .

Day 3: Hybridization

30 Wash gels 4 x 15 min with 1 mL PBS at Room temperature .

15m

30.1 Wash gels 4 x 00:15:00 in 1 mL PBS at Room temperature (1/4).

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30.2 Wash gels 4 x 00:15:00 in 1 mL PBS at Room temperature (2/4). 15m

30.3 Wash gels 4 x 00:15:00 in 1 mL PBS at Room temperature (3/4). 15m

30.4 Wash gels 4 x 00:15:00 in 1 mL PBS at Room temperature (4/4). 15m

31 DAPI stain gels for 00:10:00 with 1 mL DAPI/PBS (500 ng/ml). 10m

32 Rinse with PBS.

33 Use a dissection scope with a UV bulb to neatly trim gel edges with a razor blade.

34 Thaw and mix hybridization (hyb) and probe wash buffer. Make sure hyb buffer is clear.

35 Incubate gel in 500 µL hyb buffer for 00:30:00 @ 37 °C . 30m

36 Dilute probes 1/100 ([M] 10 Nanomolar (nM)), in 300 µL hyb buffer per gel. Vortex. Incubate @ 37 °C .

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37 Incubate gels with probes overnight @ 37 °C, no shaking necessary.

38 Put probe wash buffer and PBS @ 37 °C.

Day 4: Probe Washing

39 Wash 3 x 30 min 750 µL Probe Wash Buffer @ 37 °C.

39.1 Wash 3 x 00:30:00 750 µL Probe Wash Buffer @ 37 °C (1/3). 30m

39.2 Wash 3 x 00:30:00 750 µL Probe Wash Buffer @ 37 °C (2/3). 30m

39.3 Wash 3 x 00:30:00 750 µL Probe Wash Buffer @ 37 °C (3/3). 30m

40 Wash 3 x 30 min 1 mL PBS @ 37 °C.

40.1 Wash 3 x 00:30:00 1 mL PBS @ 37 °C (1/3). 30m

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40.2 Wash 3 x 00:30:00 1 mL PBS @ 37 °C (2/3). 30m

40.3 Wash 3 x 00:30:00 1 mL PBS @ 37 °C (3/3). 30m

41 Wash 3 x 1hr 1 mL PBS @ 37 °C.

41.1 Wash 3 x 01:00:00 1 mL PBS @ 37 °C (1/3). 1h

41.2 Wash 3 x 01:00:00 1 mL PBS @ 37 °C (2/3). 1h

41.3 Wash 3 x 01:00:00 1 mL PBS @ 37 °C (3/3). 1h

42 Keep gels in PBS at Room temperature Overnight. 1h

Day 5: Hybridization Chain Reaction (HCR)

43 30m

Incubate gels in 500 µL Amplification buffer for at least 00:30:00 @ Room temperature

44 31m 30s

Snap cool hairpins with PCR machine @ 95 °C for 00:01:30 and cool @ Room temperature for

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00:30:00 .

45 Mix hairpins h1 and h2 @ 1/100 in 300 µL Amp Buffer per gel. Vortex.

46 Incubate gel with hairpins for 03:00:00 @ Room temperature in the dark. 3h

47 Wash gels 2 x 20 min in 750 µL 5X SSCT @ Room temperature .

47.1 Wash gels 2 x 00:20:00 in 750 µL 5X SSCT @ Room temperature (1/2). 20m

47.2 Wash gels 2 x 00:20:00 in 750 µL 5X SSCT @ Room temperature (2/2). 20m

48 Wash gels 2 x 40 min in 1 mL 0.5X SSCT @ Room temperature .

48.1 Wash gels 2 x 00:40:00 in 1 mL 0.5X SSCT @ Room temperature (1/2). 40m

48.2 Wash gels 2 x 00:40:00 in 1 mL 0.5X SSCT @ Room temperature (2/2). 40m

49 Stain sample with 500 µL of anti-GFP-488 Ab @ (1/500) in PBT (0.1%) containing [M] 5 mg/mL 15m

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Ultrapure BSA and incubate Overnight (or the weekend) @ 4 °C .

Day 6: Mount and Image

50

Wash 2 x 30 min with 1 mL PBS-Triton (0.1%).

30m

50.1 Wash 2 x 00:30:00 with 1 mL PBS-Triton (0.1%) (1/2).

30m

50.2 Wash 2 x 00:30:00 with 1 mL PBS-Triton (0.1%) (2/2).

51 Wash 2 x 30 min and 1 x 1hr with 1 mL PBS.

30m

51.1 Wash 2 x 00:30:00 with 1 mL PBS (1/2).

1h

51.2 Wash 1 x 01:00:00 with 1 mL PBS (2/2).

15m

52 DAPI stain gels for 00:15:00 with 1 mL PBS/DAPI ([M] 500 ng/ml).

53

Mount gels (sample up) on an 8mm poly-lysine coated coverslip superglued to a Z.1 light-sheet sample holder and image.

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54 For gel removal from the holder, incubate gels in 750 µL of 10% Dextran Sulphate for 20 mins. Gels will shrink and fall off the coverslip.

55 For long-term storage, keep gels in 750 µL 10% Dextran Sulphate @ 4 °C

2h 45m

Stripping Probes and Hairpins for Multiplexing

30m

56 Incubate gel for 00:30:00 in 1 mL of DNAse1 Buffer @ 37 °C.

57 Add 450 µL of DNase Buffer to 50 µL DNase1. Mix.

2h

58 Incubate gel in DNase1 for 02:00:00 @ 37 °C.

59 Wash 4 x 15 min with 1 mL PBS.

15m

59.1 Wash 4 x 00:15:00 with 1 mL PBS (1/4).

15m

59.2 Wash 4 x 00:15:00 with 1 mL PBS (2/4).

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59.3 Wash 4 x 00:15:00 with 1 mL PBS (3/4). 15m

59.4 Wash 4 x 00:15:00 with 1 mL PBS (4/4). 15m

60 Hybridize with next round of probes (Day 3, step 34).

JF-669 conjugation to unlabelled hairpins 1h 45m

61 30m

Note

Turn on speed vacuum (eg. Thermofisher, SPD120) and defrost dye at Room temperature for 00:30:00.

62

Resuspend 2 mg of JF-669, SE in 630 µL Acetonitrile, mix and vortex, and aliquot in 30 µL into labelled skirted 0.5ml screw-cap centrifuge tubes > each will contain 0.1 mg of dye.

63 Evaporate acetonitrile in speed vacuum (in organic solvent mode) for 00:45:00. Can store @ -20 °C. 45m

64 In two 1.5ml Eppendorf tubes, evaporate 5 µL ([M] 500 picomolar (pM) / 10 µg) of unlabelled hairpins h1 and h2 using a speed vac, in aqueous mode, for 00:30:00. If you have 10 µL ([M] 1 nanomolar (nM)) use two tubes per hairpin. Check they have been fully evaporated. 30m

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65 Add 3 µL of [M] 0.1 Molarity (M) Sodium Bicarbonate pH 8-9 to each evaporated hairpin. Mix.

66 Add 2 µL of anhydrous DMSO to 0.1 mg of dye. Mix.

67 Add 2 µL dye mix (100 µg) to each 3 µL of hairpin (10 µg). Mix. It will change colour.

68 Leave hairpin-dye mixture to react Overnight at Room temperature in the dark.

69 The next morning, add 5 µL nuclease-free water to bring to 10 µL.

70 Remove excess dye with a QIAquick Nucleotide removal kit (add 100 µL PN1).

71 Elute dye-oligo conjugate in 50 µL nuclease-free water.

72 Check the hairpin concentration on a spectrophotometer (eg. a NanoDrop One) and dilute to [M] 60 ng/µl.

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73 Separately store hairpins h1-669 and h2-669 in 25 µL aliquot's in a PCR tube@ -20 °C.

74 Test conjugation by HCR.

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