In situ HCR v2.0 protocol for FFPE human tissue sections (*Homo sapiens sapiens*)

This protocol has been optimized for 4-μm FFPE sections.
Technical support: support@moleculartechnologies.org

Multiplexed in situ HCR protocol

Detection stage

1. Deparaffinize FFPE tissue by immersing slide in Histo-Clear II for 3 × 5 min.
   NOTE: *Each 50 mL falcon tube can fit two outward-facing slides. A volume of 30 mL is sufficient to immerse sections in the tube. If desired, a larger number of slides can be processed together using a Coplin jar.*
2. Rehydrate with a series of graded EtOH washes for 3 min at room temperature:
   (a) 100% EtOH
   (b) 100% EtOH
   (c) 95% EtOH
   (d) 70% EtOH
   (e) RNase-free H_2O
   (f) RNase-free H_2O.
3. Incubate in 1× TBS for 5 min.
4. Immerse slide in 10 μg/mL of proteinase K solution for 40 min at 37 °C.
   NOTE: *Proteinase K concentration and treatment time should be reoptimized for each batch of proteinase K.*
   NOTE: *We recommend performing this step in a falcon tube placed inside a 37 °C water bath to minimize temperature fluctuation.*
5. Wash slide 2 × 3 min at room temperature in TBST.
6. Immerse slide in 0.2 N HCl for 20 min at room temperature.
7. Incubate slides in 5× SSCT for 5 min.
8. Place slide in an RNase-free staining glass trough with a stir bar.
9. Add 200 mL of 0.1 M triethanolamine-HCl at pH 8.0.
10. Add 500 μL of acetic anhydride **slowly and carefully** with constant stirring.
    CAUTION: *Add acetic anhydride slowly with extreme care.*
11. Turn off stirrer when the acetic anhydride is dispersed and allow the slide to incubate for 10 min.
12. Incubate slide in 5× SSCT for 5 min.
13. Pre-warm two humidified chambers with one at 45 °C and the other one at 65 °C.
14. Dry slide by blotting edges on a Kimwipe.
15. Add 200 μL of probe hybridization buffer on top of the tissue sample.
    CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
16. Pre-hybridize for 10 min inside the 65 °C humidified chamber.

17. Prepare probe solution by adding 0.2 pmol of each probe (0.2 μL of 1 μM stock per probe) to 100 μL of probe hybridization buffer at 45 °C.
18. Remove the pre-hybridization solution and drain excess buffer on slide by blotting edges on a Kimwipe.
19. Add 50–100 μL of the probe solution on top of the tissue sample.
    NOTE: *Amount of probe solution depends on the size of the coverslip.*
20. Place a coverslip on the tissue sample and incubate overnight (12–16 h) in the 45 °C humidified chamber.
21. Immerse slide in 2 × SSC with 0.1% SDS at room temperature to float off coverslip.
22. Remove excess probes by incubating slide at 45 °C in:
    (a) 75% of probe wash buffer / 25% 5× SSCT for 15 min
    (b) 50% of probe wash buffer / 50% 5× SSCT for 15 min
    (c) 25% of probe wash buffer / 75% 5× SSCT for 15 min
    (d) 100% 5× SSCT for 15 min

    CAUTION: *probe wash buffer contains formamide, a hazardous material.*
    NOTE: *Wash solutions should be pre-heated to 45 °C before use.*
23. Immerse slide in 5× SSCT for 5 min at room temperature.

Amplification stage

1. Dry slide by blotting edges on a Kimwipe.
2. Add 200 μL of amplification buffer on top of the tissue sample and pre-amplify in a humidified chamber for 2 h at room temperature.
3. Separately prepare 6 pmol of hairpin H1 and 6 pmol of hairpin H2 by snap cooling 2 μL of 3 μM stock in hairpin storage buffer (heat at 95 °C for 90 seconds and cool to room temperature in a dark drawer for 30 min).
   NOTE: *HCR hairpins H1 and H2 are provided in hairpin storage buffer ready for snap cooling.*
   H1 and H2 should be snap cooled in separate tubes.
4. Prepare hairpin solution by adding all snap-cooled hairpins to 100 μL of amplification buffer at room temperature.
5. Remove the pre-amplification solution and drain excess buffer on slide by blotting edges on a Kimwipe.
6. Add 50–100 μL of the hairpin solution on top of the tissue sample.
   NOTE: *Amount of hairpin solution depends on the size of the coverslip.*
7. Place a coverslip on the tissue sample and incubate overnight (12–16 h) in a dark humidified chamber at room temperature.
8. Immerse slide in 5 × SSCT at room temperature to float off coverslip.
9. Remove excess hairpins by incubating slide in 5 × SSCT at room temperature for:
   (a) 2 × 30 min
   (b) 1 × 5 min

Sample mounting for microscopy

1. Dry slide by blotting edges on a Kimwipe.
2. Add 50 μL of SlowFade Gold antifade mountant with DAPI on top of human tissue section.
3. Place a 22 mm $\times$ 40 mm No. 1 coverslip on top and seal using clear nail polish.
4. A Zeiss Axio Observer Z1 fluorescent inverted microscope equipped with an Plan-Apochromat 63$\times$/1.4 Oil DIC M27 objective was used in our lab to acquire tissue section images. Samples were illuminated with X-Cite Series 120 (EXFO Photonics) and images were collected with an ImagEM-1K EM-CCD digital camera (Hamamatsu).

Buffer recipes for in situ HCR v2.0

Probes, amplifiers, probe hybridization buffer, and probe wash buffer should be stored at -20 °C. Amplification buffer should be stored at 4 °C. Keep these reagents on ice at all times during probe and amplifier preparation. Make sure all solutions are well mixed before use.

Proteinase K solution	For 30 mL of solution
10 μg/mL proteinase K	15 μL of 20 mg/mL proteinase K Fill up to 30 mL with 1× TBS
0.2 N HCl	For 30 mL of solution
0.2 N HCl	500 μL of 37% ($\approx$ 12 N) HCl Fill up to 30 mL with ultrapure H_2O
0.1 M Triethanolamine-HCl	For 200 mL of solution
0.1 M triethanolamine Adjust pH to 8.0	2.67 mL of 7.5 M triethanolamine 350 μL 37% HCl Fill up to 200 mL with ultrapure H_2O
TBST	For 50 mL of solution
1× TBS 0.1% Tween 20	5 mL of 10× TBS 500 μL of 10% Tween 20 Fill up to 50 mL with ultrapure H_2O
Probe hybridization buffer	For 40 mL of solution
50% formamide 5× sodium chloride sodium citrate (SSC) 9 mM citric acid (pH 6.0) 0.1% Tween 20 50 μg/mL heparin 1× Denhardt's solution 10% dextran sulfate	20 mL formamide 10 mL of 20× SSC 360 μL 1 M citric acid, pH 6.0 400 μL of 10% Tween 20 200 μL of 10 mg/mL heparin 800 μL of 50× Denhardt's solution 8 mL of 50% dextran sulfate Fill up to 40 mL with ultrapure H_2O
Probe wash buffer	For 40 mL of solution
50% formamide 5× sodium chloride sodium citrate (SSC) 9 mM citric acid (pH 6.0) 0.1% Tween 20 50 μg/mL heparin	20 mL formamide 10 mL of 20× SSC 360 μL 1 M citric acid, pH 6.0 400 μL of 10% Tween 20 200 μL of 10 mg/mL heparin Fill up to 40 mL with ultrapure H_2O
Amplification buffer	For 40 mL of solution
5× sodium chloride sodium citrate (SSC) 0.1% Tween 20 10% dextran sulfate 100 μg/mL salmon sperm DNA	10 mL of 20× SSC 400 μL of 10% Tween 20 8 mL of 50% dextran sulfate 400 μL of 10 mg/mL salmon sperm DNA Fill up to 40 mL with ultrapure H_2O

5× SSCT

5× sodium chloride sodium citrate (SSC)
0.1% Tween 20

For 40 mL of solution
10 mL of 20× SSC
400 μL of 10% Tween 20
Fill up to 40 mL with ultrapure H_2O

50% dextran sulfate

50% dextran sulfate

For 40 mL of solution
20 g of dextran sulfate powder
Fill up to 40 mL with ultrapure H_2O

Reagents and supplies

Histo-Clear II (National Diagnostics Cat. # HS-202)
Ethanol, 200 proof (VWR Cat. # V1001G)
10× Tris-buffered saline solution (TBS) (Research Products International Cat. # T60075)
20 mg/mL Proteinase K (Life Technologies Cat. # AM2546)
10% Tween 20 Solution (Bio-Rad Cat. # 161-0781)
Hydrochloric Acid (HCl) (EMD Millipore Cat. # HX0603-75)
Triethanolamine (Acros Organics Cat. # AC42163-1000)
Acetic anhydride (Mallinckrodt Chemicals Cat. # 2420-04) Formamide (Deionized) (Ambion Cat. # AM9342)
20× sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)
Heparin (Sigma Cat. # H3393)
50× Denhardt's solution (Life Technologies Cat. # 750018)
Dextran sulfate, mol. wt. $>$ 500,000 (Sigma Cat. # D6001)
UltraPure salmon sperm DNA solution (Life Technologies Cat. #15632-011)
22 mm × 30 mm No. 1 coverslip (VWR Cat. # 48393-026)
SlowFade Gold antifade mountant with DAPI (Life Technologies Cat. # S36938)

Citation Notes

Molecular Technologies (moleculartechnologies.org) is a non-profit academic resource within the Beckman Institute at Caltech. For citation, please select from the list below as appropriate for your application:

- **In situ HCR v3.0**
  Automatic background suppression for dramatically enhanced performance (signal-to-background and quantitative precision) and ease-of-use (no probe set optimization for new targets) (Choi *et al.*, 2018). v3.0 supports three quantitative modes: qHCR imaging – analog mRNA relative quantitation with subcellular resolution; qHCR flow cytometry – analog mRNA relative quantitation for high-throughput single-cell analysis; dHCR imaging – digital mRNA absolute quantitation. Protocols for v3.0 in diverse organisms are adapted from the zoo paper.

- **Quantitative HCR (qHCR) imaging**
  mRNA relative quantitation with subcellular resolution in the anatomical context of whole-mount vertebrate embryos; read-out/read-in analysis framework (Trivedi *et al.*, 2018).

- **Zoo paper**
  Protocols for multiplexed mRNA imaging in diverse sample types (Choi *et al.*, 2016):

bacteria in suspension	whole-mount zebrafish embryos and larvae
whole-mount fruit fly embryos	whole-mount chicken embryos
whole-mount worm larvae	whole-mount mouse embryos
whole-mount sea urchin embryos	FFPE human tissue sections

- **Single-molecule HCR imaging**
  Single-molecule mRNA imaging in thick autofluorescent samples (e.g., 0.5 mm adult mouse brain sections) (Shah *et al.*, 2016).

- **Multiplexed quantitative HCR (qHCR) northern blots**
  Simultaneous quantification of RNA target size and abundance with signal amplification for up to 5 target RNAs (Schwarzkopf & Pierce, 2016).

- **In situ HCR v2.0**
  2nd generation in situ HCR technology (v2.0) using DNA probes and DNA HCR amplifiers: 10× increase in signal, 10× reduction in cost, dramatic increase in reagent durability (Choi *et al.*, 2014).

- **Shielded covalent (SC) probes**
  Highly selective covalent capture of DNA and RNA targets, overcoming the longstanding tradeoff between selectivity and durable target capture (Vieregg *et al.*, 2013).

- **In situ HCR v1.0**
  1st generation in situ HCR technology (v1.0) using RNA probes and RNA HCR amplifiers: multiplexed mRNA imaging in whole-mount vertebrate embryos with simultaneous signal amplification for up to 5 target mRNAs (Choi *et al.*, 2010).

- **Hybridization chain reaction (HCR) mechanism**
  Isothermal enzyme-free molecular signal amplification (Dirks & Pierce, 2004).

We are happy to provide advice and technical support: support@moleculartechnologies.org.

– The Molecular Technologies Team

References

Choi, H. M. T., Chang, J. Y., Trinh, L. A., Padilla, J. E., Fraser, S. E., & Pierce, N. A. (2010). Programmable in situ amplification for multiplexed imaging of mRNA expression. *Nat Biotechnol*, **28**(11), 1208–12. (pdf, supp info, supp movie 1, supp movie 2)

Choi, H. M. T., Beck, V. A., & Pierce, N. A. (2014). Next-generation in situ hybridization chain reaction: higher gain, lower cost, greater durability. *ACS Nano*, **8**(5), 4284–4294. (pdf, supp info, supp movie)

Choi, H. M. T., Calvert, C. R., Husain, N., Huss, D., Barsi, J. C., Deverman, B. E., Hunter, R. C., Kato, M., Lee, S. M., Abelin, A. C. T., Rosenthal, A. Z., Akbari, O. S., Li, Y., Hay, B. A., Sternberg, P. W., Patterson, P. H., Davidson, E. H., Mazmanian, S. K., Prober, D. A., van de Rijn, M., Leadbetter, J. R., Newman, D. K., Readhead, C., Bronner, M. E., Wold, B., Lansford, R., Sauka-Spengler, T., Fraser, S. E., & Pierce, N. A. (2016). Mapping a multiplexed zoo of mRNA expression. *Development*, **143**, 3632–3637. (pdf, supp info, fruit fly movie, sea urchin movie, zebrafish movie, chicken movie, mouse movie)

Choi, H. M. T., Schwarzkopf, M., Fornace, M. E., Acharya, A., Artavanis, G., Stegmaier, J., Cunha, A., & Pierce, N. A. (2018). Third-generation in situ hybridization chain reaction: multiplexed, quantitative, sensitive, versatile, robust. *bioRxiv*, doi: http://dx.doi.org/10.1101/285213. (pdf, supp info)

Dirks, R. M., & Pierce, N. A. (2004). Triggered amplification by hybridization chain reaction. *Proc Natl Acad Sci USA*, **101**(43), 15275–15278. (pdf)

Schwarzkopf, M., & Pierce, N. A. (2016). Multiplexed miRNA northern blots via hybridization chain reaction. *Nucleic Acids Res*, **44**(15), e129. (pdf, supp info)

Shah, S., Lubeck, E., Schwarzkopf, M., He, T.-F., Greenbaum, A., Sohn, C. H., Lignell, A., Choi, H. M. T., Gradinaru, V., Pierce, N. A., & Cai, L. (2016). Single-molecule RNA detection at depth via hybridization chain reaction and tissue hydrogel embedding and clearing. *Development*, **143**, 2862–2867. (pdf, supp info, supp movie 1, supp movie 2, supp movie 3)

Trivedi, V., Choi, H. M. T., Fraser, S. E., & Pierce, N. A. (2018). Multidimensional quantitative analysis of mRNA expression within intact vertebrate embryos. *Development*, **145**, doi:10.1242/dev.156869. (pdf, supp info, Read-out/Read-in 1.0 package)

Vieregg, J. R., Nelson, H. M., Stoltz, B. M., & Pierce, N. A. (2013). Selective nucleic acid capture with shielded covalent probes. *J Am Chem Soc*, **135**(26), 9691–9699. (pdf, supp info)