In situ HCR v2.0 protocol for bacteria in suspension (*Escherichia coli*)

This protocol has not been validated for all types of bacteria and should only be used as a template.
Technical support: support@moleculartechnologies.org

Sample preparation

1. Grow *E. coli* cells (from frozen glycerol stock) in 2 mL of LB media overnight in a 37 °C shaker.
2. Dilute to make a 5 mL liquid culture with OD_{600} = 0.05.
3. Incubate in a 37 °C shaker until $OD_{600} \approx 0.5$ (exponential phase).
4. Aliquot 1 mL of cells and centrifuge for 10 min.
   NOTE: *Centrifugation should be as gentle as possible to pellet cells. For E. coli, all centrifugation steps are done at 4000 × g.*
5. Remove supernatant and re-suspend cells in 750 μL of 1× phosphate-buffered saline (PBS).
6. Add 250 μL of 4% formaldehyde and incubate overnight at 4 °C.
   CAUTION: *Use formaldehyde with extreme care as it is a hazardous material.*
7. Centrifuge for 10 min and remove supernatant.
8. Re-suspend cells in 150 μL of 1× PBS.
9. Add 850 μL of 100% MeOH and store cells at -20 °C before use.

Buffer recipes for sample preparation

LB media
5 g of Novagen LB Broth Miller powder
Fill up to 200 mL with ultrapure H_2O
Autoclave at 121 °C for 20 min

4% formaldehyde
4% formaldehyde
1× PBS

For 10 mL of solution
2.5 mL of 16% formaldehyde
1 mL of 10× PBS
Fill up to 10 mL with ultrapure H_2O

NOTE: *Avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the samples.*

Multiplexed in situ HCR v2.0 protocol

Detection stage

1. Transfer 100 μL of cells into a 1.5 mL eppendorf tube.
2. Centrifuge for 5 min and remove supernatant.
3. Wash cells with 500 μL of 1$\times$ PBST and remove the solution by centrifugation.
4. Re-suspend the pellet with 400 μL of probe hybridization buffer and pre-hybridize for 1 h at 65 °C.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
   NOTE: *We pre-hybridize for 1h at 65 °C to alleviate signal from fluorescent proteins; we pre-hybridize for 1 h at 45 °C if this is not a consideration.*
5. In the meantime, prepare probe mixture by adding 1 pmol of each probe (1 μL of 1 μM stock per probe) to 100 μL of probe hybridization buffer pre-heated to 45 °C.
6. Add the probe mixture directly to the sample to reach a final probe concentration of 2 nM.
7. Incubate the sample for 2 h at 45 °C.
8. Add 1 mL of probe wash buffer (pre-heated to 45 °C) to the sample to dilute the probes.
   CAUTION: *probe wash buffer contains formamide, a hazardous material.*
9. Centrifuge for 5 min and remove the wash solution.
10. Re-suspend the cell pellet with 500 μL of probe wash buffer (pre-heated to 45 °C).
11. Incubate for 5 min at 45 °C and remove the wash solution by centrifugation for 5 min.
12. Repeat steps 10 and 11 for two additional times but with 10 min incubation.
13. Proceed to hairpin amplification.

Amplification stage

1. Re-suspend the cell pellet with 250 μL of amplification buffer and pre-amplify for 10 min at room temperature.
2. Separately prepare 30 pmol of hairpin H1 and 30 pmol of hairpin H2 by snap cooling 10 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.
3. Prepare hairpin mixture by adding all snap-cooled hairpins to 250 μL of amplification buffer at room temperature.
4. Add the hairpin mixture directly to the sample to reach a final hairpin concentration of 60 nM.
5. Incubate the sample overnight ($>$12 h) in the dark at room temperature.
6. Add 1 mL of 5$\times$ SSCT at room temperature to the sample to dilute the hairpins.
7. Centrifuge for 5 min and remove the wash solution.
8. Re-suspend the cell pellet with 500 μL of 5$\times$ SSCT.
9. Incubate for 5 min at room temperature and remove the wash solution by centrifugation for 5 min.

10. Repeat steps 8 and 9 for two additional times but with 10 min incubation.
11. Re-suspend the cell pellet in 50 μL of 5$\times$ SSCT.
12. Store the sample at 4 °C before imaging.

Sample mounting for microscopy

1. Add 50 μL of SlowFade Gold antifade mountant to each sample.
2. Pipette 50 μL of 0.1% (w/v) poly-L-lysine onto a 22 mm $\times$ 22 mm coverslip.
3. Allow to sit for 10 min and tap off extra solution. Allow it to air dry at room temperature.
4. Add 10 μL of sample to the middle of the coverslip.
5. Place the sample side of the coverslip on top of a 25 mm $\times$ 75 mm glass slide. Lower the coverslip carefully to avoid air bubbles.
6. Sample slide could be imaged on a wide-field fluorescent microscope. A Zeiss Axio Observer Z1 fluorescent inverted microscope equipped with an Plan-Apochromat 100$\times$/1.4 Oil DIC objective was used in our lab to acquire bacterial 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.

Probe hybridization buffer (low MW dextran sulfate)	For 40 mL of solution
50% formamide	20 mL formamide
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
9 mM citric acid (pH 6.0)	360 μL 1 M citric acid, pH 6.0
0.1% Tween 20	400 μL of 10% Tween 20
50 μg/mL heparin	200 μL of 10 mg/mL heparin
1× Denhardt's solution	800 μL of 50× Denhardt's solution
10% low MW dextran sulfate	8 mL of 50% low MW dextran sulfate
	Fill up to 40 mL with ultrapure H_2O

Probe wash buffer	For 40 mL of solution
50% formamide	20 mL formamide
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
9 mM citric acid (pH 6.0)	360 μL 1 M citric acid, pH 6.0
0.1% Tween 20	400 μL of 10% Tween 20
50 μg/mL heparin	200 μL of 10 mg/mL heparin
	Fill up to 40 mL with ultrapure H_2O

Amplification buffer (low MW dextran sulfate)	For 40 mL of solution
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
0.1% Tween 20	400 μL of 10% Tween 20
10% low MW dextran sulfate	8 mL of 50% low MW dextran sulfate
	Fill up to 40 mL with ultrapure H_2O

Hairpin storage buffer	For 40 mL of solution
10 mM Tris	400 μL of 1 M Tris
1 mM EDTA	80 μL of 0.5 M EDTA
300 mM NaCl	4 mL of 3 M NaCl
	Fill up to 40 mL with ultrapure H_2O

5× SSCT	For 40 mL of solution
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
0.1% Tween 20	400 μL of 10% Tween 20
	fill up to 40 mL with ultrapure H_2O

50% dextran sulfate	For 40 mL of solution
50% dextran sulfate	20 g of low MW dextran sulfate powder
	Fill up to 40 mL with ultrapure H_2O

Reagents and supplies

LB Broth Miller (Novagen Cat. # 71753-5)
10× PBS (Life Technologies Cat.# AM9625)
Methanol (Mallinckrodt Chemicals Cat. # 3016-16)
Formaldehyde, 16%, methanol free, Ultra Pure (Polyysciences Cat. # 18814-20)
Formamide (Deionized) (Ambion Cat. # AM9342)
20× sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)
Heparin (Sigma Cat. # H3393)
50% Tween 20 (Life Technologies Cat. # 00-3005)
50× Denhardt's solution (Life Technologies Cat. # 750018)
Dextran sulfate, MW 6,500-10,000 (Sigma Cat. # D4911)
25 mm × 75 mm glass slide (VWR Cat. # 48300-025)
22 mm × 22 mm No. 1 coverslip (VWR Cat. # 48366-067)
SlowFade Gold antifade mountant (Life Technologies Cat. # S36936)

Citation Notes

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- **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)