Molecular Technologies
MAMMALIAN CELLS IN SUSPENSION

# In situ HCR v3.0 protocol for mammalian cells in suspension

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

## Sample preparation protocol

1. Aspirate growth media from culture plate and wash cells with DPBS.
   NOTE: *Avoid using calcium chloride and magnesium chloride in DPBS as this leads to increased autofluorescence.*
2. Add 3 mL of trypsin per 10 cm plate and incubate in a 5% $CO_2$ incubator at 37 °C for 5 min.
3. Quench trypsin by adding 3 mL of growth media.
4. Transfer cells to a 15 mL conical tube and centrifuge for 5 min at 180 × g.
5. Aspirate supernatant and re-suspend cells in 4% formaldehyde to reach approximately $10^6$ cells/mL.
   CAUTION: *Use formaldehyde with extreme care as it is a hazardous material.*
6. Fix cells for at least 1 hr at room temperature.
7. Centrifuge for 5 min at 180 × g and aspirate supernatant.
8. Wash cells 4 times with PBST (use the same volume as formaldehyde solution).
9. Re-suspend cells in cold 70% ethanol (use the same volume as formaldehyde and PBST solutions).
10. Store cells at 4 °C overnight before use.

## Buffer recipes for sample preparation

| **4% formaldehyde in PBST** | For 10 mL of solution |
|---|---|
| 4% formaldehyde | 2.5 mL of 16% formaldehyde |
| 1× PBS | 1 mL of 10× PBS |
| 0.1% Tween 20 | 100 $\mu$L of 10% Tween 20 |
| | 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 v3.0 protocol

### Detection stage

1. Transfer desired amount (0.5-1 $\times$ $10^6$) of fixed cells into a 1.5 mL eppendorf tube.
2. Centrifuge for 5 min and remove supernatant.
   NOTE: *Centrifugation should be as gentle as possible to pellet cells. All centrifugation steps are done at 180 $\times$ g.*
3. Wash cells twice with 500 $\mu$L of PBST. Centrifuge for 5 min to remove supernatant.
4. Re-suspend the pellet with 400 $\mu$L of 30% probe hybridization buffer and pre-hybridize for 30 min at 37 °C.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
5. In the meantime, prepare probe solution by adding 2 pmol of each probe mixture (odd & even: 1 $\mu$L of 2 $\mu$M stock per probe mixture) to 100 $\mu$L of 30% probe hybridization buffer pre-heated to 37 °C.
6. Add the probe solution directly to the sample to reach a final probe concentration of 4 nM.
   NOTE: *For dHCR imaging, use 3 pmol of probe to improve probe hybridization efficiency.*
7. Incubate the sample overnight at 37 °C.
8. Centrifuge for 5 min to remove probe solution.
9. Re-suspend the cell pellet with 500 $\mu$L of 30% probe wash buffer.
   CAUTION: *probe wash buffer contains formamide, a hazardous material.*
   NOTE: *Wash solutions should be pre-heated to 37°C before use.*
10. Incubate for 10 min at 37 °C and remove the wash solution by centrifugation for 5 min.
11. Repeat steps 9 and 10 for three additional times.
12. Re-suspend the cell pellet with 500 $\mu$L of 5$\times$ SSCT.
13. Incubate for 5 min at room temperature.
14. Proceed to hairpin amplification.

### Amplification stage

1. Centrifuge for 5 min to pellet the cells.
2. Re-suspend the cell pellet with 150 $\mu$L of amplification buffer and pre-amplify for 30 min at room temperature.
3. Separately prepare 15 pmol of hairpin H1 and 15 pmol of hairpin H2 by snap cooling 5 $\mu$L of 3 $\mu$M stock (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 mixture by adding all snap-cooled hairpins to 100 $\mu$L of amplification buffer at room temperature.
5. Add the hairpin mixture directly to the sample to reach a final hairpin concentration of 60 nM.
6. Incubate the sample overnight (>12 h) in the dark at room temperature.
   NOTE: *For dHCR imaging, amplify for 45-min to ensure single-molecule dots are diffraction-limited.*

7. Centrifuge for 5 min and remove the hairpin solution.
8. Re-suspend the cell pellet with 500 $\mu$L of 5$\times$ SSCT.
9. Without incubation, remove the wash solution by centrifugation for 5 min.
10. Repeat steps 8 and 9 for five additional times.
11. Re-suspend the cell pellet in desired buffer and volume.
12. Filter cells before flow cytometry.

## Buffer recipes for in situ HCR

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.

| **30% probe hyb buffer (low MW dextran sulfate)** | For 40 mL of solution |
|---|---|
| 30% formamide | 12 mL formamide |
| 5× sodium chloride sodium citrate (SSC) | 10 mL of 20× SSC |
| 9 mM citric acid (pH 6.0) | 360 $\mu$L 1 M citric acid, pH 6.0 |
| 0.1% Tween 20 | 400 $\mu$L of 10% Tween 20 |
| 50 $\mu$g/mL heparin | 200 $\mu$L of 10 mg/mL heparin |
| 1× Denhardt's solution | 800 $\mu$L 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 $H_2O$ |

| **30% probe wash buffer** | For 40 mL of solution |
|---|---|
| 30% formamide | 12 mL formamide |
| 5× sodium chloride sodium citrate (SSC) | 10 mL of 20× SSC |
| 9 mM citric acid (pH 6.0) | 360 $\mu$L 1 M citric acid, pH 6.0 |
| 0.1% Tween 20 | 400 $\mu$L of 10% Tween 20 |
| 50 $\mu$g/mL heparin | 200 $\mu$L 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 $\mu$L of 10% Tween 20 |
| 10% low MW dextran sulfate | 8 mL of 50% low MW dextran sulfate |
| | Fill up to 40 mL with ultrapure $H_2O$ |

| **5× SSCT** | For 40 mL of solution |
|---|---|
| 5× sodium chloride sodium citrate (SSC) | 10 mL of 20× SSC |
| 0.1% Tween 20 | 400 $\mu$L 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**

DPBS, no calcium, no magnesium (Life Technologies Cat. # 14190144)
Trypsin-EDTA (0.25%), phenol red (Life Technologies Cat. # 25200072)
16% Formaldehyde (w/v), methanol-free (Life Technologies Cat. # 28908)
10× PBST (Rockland Cat. # MB-075-1000)
10% Tween 20 solution (Bio-Rad Cat. # 161-0781)
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, mol. wt. 6,500-10,000 (Sigma Cat. # D4911)

## 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. *Development*, **145**, doi:10.1242/dev.165753. (pdf, supp info, Dot Analysis 1.0 package)

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)