HCR 2°IHC protocol for mammalian cells on a chambered slide

This protocol has not been validated for all mammalian cell types and should only be used as a template.

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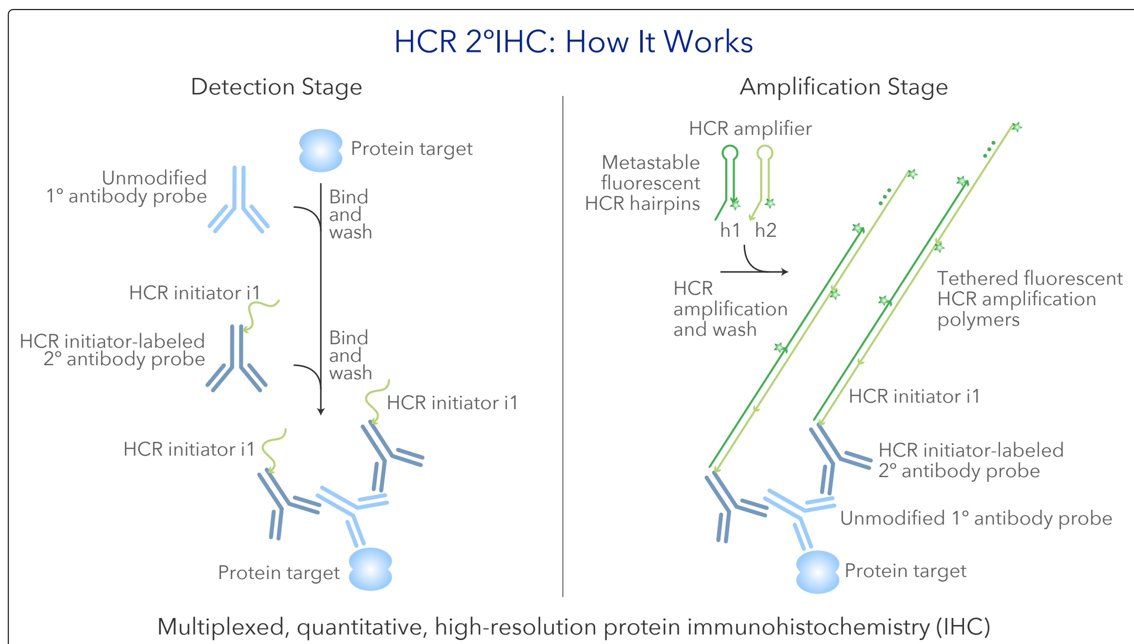

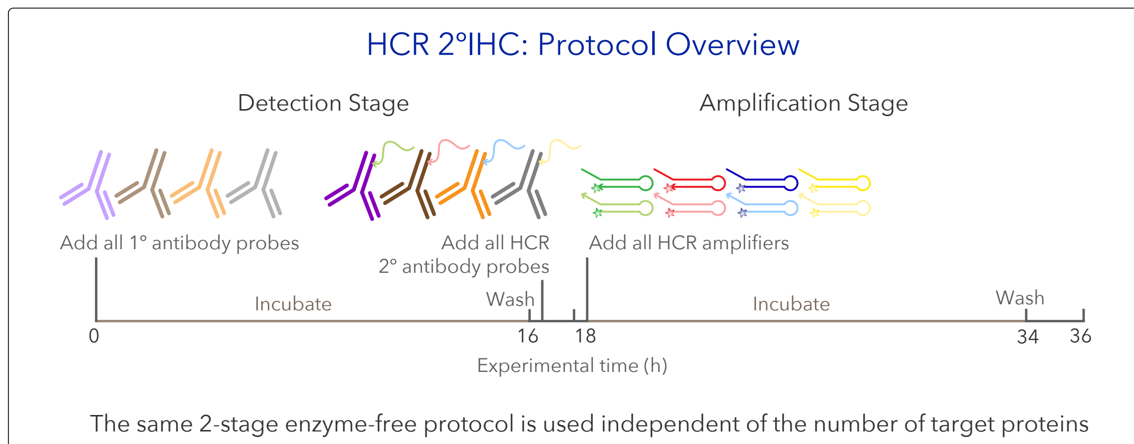

HCR 2°IHC

Multiplexed, quantitative, high-resolution protein imaging

Multiplexed Experiment

- Order one HCR IHC bundle per target protein

Example 2-Plex Experiment

- HCR bundle for target Protein1
  - 1° Ab: Rabbit Anti-Protein1 (your own 1° antibody)
  - HCR 2° Ab probe: Donkey Anti-Rabbit for use with amplifier B1
  - HCR amplifier: B1-647
  - HCR IHC buffers: antibody buffer, amplification buffer (for use with all bundles)

- HCR bundle for target Protein2
  - 1° Ab: Mouse Anti-Protein2 (your own 1° antibody)
  - HCR 2° Ab probe: Donkey Anti-Mouse for use with amplifier B2
  - HCR amplifier: B2-488

Storage conditions

- Store HCR antibody probes and HCR amplifiers at -20 °C.
- Store HCR antibody buffer and HCR amplification buffer at 4 °C.
- On the bench top, keep stock solutions on ice.
- Make sure all solutions are well mixed before use.

Preparation of fixed mammalian cells on a chambered slide

1. Coat bottom of each chamber by applying 300 μL of 0.01% poly-D-lysine prepared in cell culture grade H_2O.
   NOTE: *A volume of 300 μL is sufficient per chamber on an 8-chamber slide. Scale volume accordingly if using a different slide format.*
2. Incubate for at least 30 min at room temperature.
3. Aspirate the coating solution and wash each chamber twice with molecular biology grade H_2O.
4. Plate desired number of cells in each chamber.
5. Grow cells to desired confluency for 24–48 h.
6. Aspirate growth media and wash each chamber with 300 μL of DPBS.
   NOTE: *avoid using calcium chloride and magnesium chloride in DPBS as this leads to increased autofluorescence.*
7. Add 300 μL of 4% formaldehyde to each chamber.
   CAUTION: *use formaldehyde with extreme care as it is a hazardous material.*
8. Incubate for 10 min at room temperature.
9. Remove fixative and wash each chamber 2 $\times$ 300 μL of DPBS.
10. Aspirate DPBS and add 300 μL of ice-cold 70% ethanol (EtOH).
11. Permeabilize cells overnight (or longer) at -20 °C.
12. Proceed to HCR assay.

Multiplexed HCR 2°IHC protocol

Detection stage

1. Aspirate EtOH from sample and wash samples 2 × 5 min with 300 μL of 1× PBS.
2. Apply 300 μL antibody buffer to each chamber. Incubate at room temperature for 1 hr with gentle agitation.
3. Prepare working concentration of primary antibodies in antibody buffer. Prepare 300 μL per chamber.
   NOTE: *follow manufacturer's guidelines for primary antibody working concentration.*
4. Replace antibody buffer with primary antibody solution and incubate overnight (>12 h) at 4 °C with gentle agitation.
   NOTE: *Incubation may be optimized (e.g., 1–2 h at room temperature) depending on sample type and thickness.*
5. Remove excess antibodies by washing 3 × 5 min with 1× PBST at room temperature with gentle agitation.
6. Prepare 1 μg/mL working concentration of initiator-labeled secondary antibodies in antibody buffer. Prepare 300 μL per chamber.
   NOTE: *Concentration may be optimized depending on protein target and primary antibody.*
7. Add secondary antibody solution to each chamber and incubate 1 h at room temperature with gentle agitation.
8. Remove excess antibodies by washing 3 × 5 min with 1× PBST at room temperature with gentle agitation.

Amplification stage

1. Wash with 300 μL 5× SSCT at room temperature for 5 min.
2. Pre-amplify samples in 300 μL of amplification buffer for 30 min at room temperature.
   NOTE: *Equilibrate amplification buffer to room temperature before use.*
3. Separately prepare 18 pmol of hairpin h1 and 18 pmol of hairpin h2 by snap cooling 6 μL of 3 μM 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. This is the amount of hairpins needed for each target in a single sample using 300 μL of incubation volume.*
4. Prepare a 60 nM hairpin solution by adding all snap-cooled h1 hairpins and snap-cooled h2 hairpins to 300 μL of amplification buffer at room temperature per sample.
5. Remove the pre-amplification solution and add the hairpin solution.
6. Incubate the slide overnight (>12 h) protected from light at room temperature.
7. Remove excess hairpins by washing 5 × 5 min with 300 μL of 5× SSCT at room temperature.
8. Remove final wash and add 150 μL of mounting medium.
9. Slides can be stored at 4 °C protected from light prior to imaging.

Buffer recipes

4% formaldehyde in PBS	For 10 mL of solution
4% formaldehyde	2.5 mL of 16% formaldehyde
1× PBS	1 mL of 10× PBS
	Fill up to 10 mL with molecular biology grade H_2O

1× PBST	For 40 mL of solution
1× phosphate buffered solution (PBS)	4 mL of 10× PBS
0.1% Tween 20	400 μL of 10% Tween 20
	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

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

HCR Technology Citation Notes

For citation, please select from the list below as appropriate for your application:

- **HCR IHC + HCR RNA-FISH**
  HCR IHC + HCR RNA-FISH enables a unified approach to multiplexed, quantitative, high-resolution protein immunohistochemistry (IHC) and RNA fluorescence in situ hybridization (RNA-FISH), with quantitative 1-step enzyme-free HCR signal amplification performed for all protein and RNA targets simultaneously (Schwarzkopf et al., 2021).

- **HCR IHC**
  HCR IHC enables multiplexed, quantitative, high-resolution protein immunohistochemistry (IHC) in highly autofluorescent samples (e.g., FFPE brain tissue sections) (Schwarzkopf et al., 2021).

- **HCR RNA-FISH (v3.0)**
  Third-generation HCR RNA-FISH (v3.0) enables multiplexed, quantitative, high-resolution RNA fluorescence in situ hybridization (RNA-FISH) with automatic background suppression *throughout the protocol* for dramatically enhanced performance (signal-to-background, qHCR precision, dHCR fidelity) and ease-of-use (no probe set optimization for new targets and organisms) (Choi et al., 2018). Quantitative analysis modes:

  - qHCR RNA imaging: analog mRNA relative quantitation with subcellular resolution in the anatomical context of thick autofluorescent samples.
  - dHCR RNA imaging: digital mRNA absolute quantitation with single-molecule resolution in the anatomical context of thick autofluorescent samples.
  - qHCR RNA flow cytometry: analog mRNA relative quantitation for high-throughput expression profiling of mammalian cells and bacteria.

  Protocols for HCR RNA-FISH (v3.0) in diverse organisms are adapted from the Zoo paper.

- **qHCR RNA imaging**
  qHCR RNA imaging enables mRNA relative quantitation with subcellular resolution in the anatomical context of thick autofluorescent samples (e.g., whole-mount vertebrate embryos). The read-out/read-in analysis framework enables bidirectional quantitative discovery in an anatomical context (Trivedi et al., 2018).

- **Zoo paper**
  Protocols for multiplexed mRNA imaging in diverse sample types (Choi et al., 2016):
  - bacteria in suspension
  - FFPE human tissue sections
  - generic sample in solution
  - generic sample on a slide
  - mammalian cells on a slide
  - mammalian cells in suspension
  - whole-mount chicken embryos
  - whole-mount fruit fly embryos
  - whole-mount mouse embryos
  - whole-mount nematode larvae
  - whole-mount sea urchin embryos
  - whole-mount zebrafish embryos and larvae

- **dHCR imaging**
  dHCR RNA imaging enables RNA absolute quantitation with single-molecule resolution in the anatomical context of thick autofluorescent samples (e.g., 0.5 mm adult mouse brain sections) (Shah et al., 2016).

- **qHCR northern blots**
  qHCR northern blots enable multiplexed quantification of RNA target size and abundance for up to 5 target RNAs (Schwarzkopf & Pierce, 2016).

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

- **HCR RNA-FISH (v1.0)**
  First-generation HCR RNA-FISH technology (v1.0) using RNA HCR 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).

- **HCR mechanism**
  The hybridization chain reaction (HCR) mechanism enables multiplexed, quantitative, 1-step, isothermal, enzyme-free signal amplification in diverse technological settings (Dirks & Pierce, 2004).

HCR Technology References

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