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TECHNIQUES AND RESOURCES

RESEARCH ARTICLE

Third-generation *in situ* hybridization chain reaction: multiplexed, quantitative, sensitive, versatile, robust

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ABSTRACT

In situ hybridization based on the mechanism of the hybridization chain reaction (HCR) has addressed multi-decade challenges that impeded imaging of mRNA expression in diverse organisms, offering a unique combination of multiplexing, quantitation, sensitivity, resolution and versatility. Here, with third-generation *in situ* HCR, we augment these capabilities using probes and amplifiers that combine to provide automatic background suppression throughout the protocol, ensuring that reagents will not generate amplified background even if they bind non-specifically within the sample. Automatic background suppression dramatically enhances performance and robustness, combining the benefits of a higher signal-to-background ratio with the convenience of using unoptimized probe sets for new targets and organisms. *In situ* HCR v3.0 enables three multiplexed quantitative analysis modes: (1) qHCR imaging – analog mRNA relative quantitation with subcellular resolution in the anatomical context of whole-mount vertebrate embryos; (2) qHCR flow cytometry – analog mRNA relative quantitation for high-throughput expression profiling of mammalian and bacterial cells; and (3) dHCR imaging – digital mRNA absolute quantitation via single-molecule imaging in thick autofluorescent samples.

KEY WORDS: Automatic background suppression, dHCR imaging, *In situ* HCR v3.0, Multiplexed quantitative *in situ* hybridization, qHCR flow cytometry, qHCR imaging

INTRODUCTION

HCR provides isothermal enzyme-free signal amplification in diverse technological settings *in vitro*, *in situ* and *in vivo* (Ikbal et al., 2015; Bi et al., 2017). Each HCR amplifier consists of two species of kinetically trapped DNA hairpins (H1 and H2; Fig. 1A) that co-exist metastably on lab time scales, storing the energy to drive a conditional self-assembly cascade upon exposure to a cognate DNA initiator sequence (I1) (Dirks and Pierce, 2004; Choi et al., 2014). Initiator I1 hybridizes to the input domain of hairpin H1, opening the hairpin to expose its output domain, which in turn hybridizes to the input domain of hairpin H2, exposing its output domain which is identical in sequence to initiator I1, thus providing the basis for a chain reaction of alternating H1 and H2 polymerization steps.

In the context of fluorescence *in situ* hybridization experiments, where the objective is to image mRNA expression patterns within fixed biological specimens, the role of HCR *in situ* amplification is to boost the signal above background autofluorescence inherent to the sample. Using *in situ* HCR v2.0, the initiator I1 is appended to DNA probes complementary to a target mRNA of interest, triggering the self-assembly of fluorophore-labeled H1 and H2 hairpins into tethered fluorescent amplification polymers (Choi et al., 2014, 2016; Shah et al., 2016a; Trivedi et al., 2018). *In situ* HCR v2.0 enables state-of-the-art mRNA imaging in challenging imaging settings (Choi et al., 2016), including whole-mount vertebrate embryos and thick tissue sections, offering three unique capabilities: straightforward multiplexing with simultaneous one-stage signal amplification for up to five targets (Choi et al., 2014), analog mRNA relative quantitation in an anatomical context (qHCR imaging) (Trivedi et al., 2018), digital mRNA absolute quantitation in an anatomical context (dHCR imaging) (Shah et al., 2016a).

Using *in situ* HCR v2.0, each target mRNA is detected using multiple probes each carrying a full HCR initiator I1 (Fig. 1B, left). If a probe binds non-specifically within the sample, initiator I1 will nonetheless trigger HCR, generating amplified background that decreases the signal-to-background ratio of the image. As a result, using *in situ* HCR v2.0, it is crucial to use probe sets that exclude probes that bind non-specifically, sometimes necessitating probe set optimization in which probes are tested individually to remove 'bad probes'. To enhance robustness and eliminate the potential need for probe set optimization when exploring new targets, *in situ* HCR v3.0 employs probe and amplifier concepts that combine to achieve automatic background suppression throughout the protocol, ensuring that even if a reagent binds non-specifically within the sample, it will not lead to generation of amplified background.

Automatic background suppression is inherent to HCR hairpins because polymerization is conditional on the presence of the initiator I1; individual H1 or H2 hairpins that bind non-specifically in the sample do not trigger formation of an amplification polymer. Hence, the needed innovation is a probe concept that will generate initiator I1 conditionally upon detection of the target mRNA. *In situ* HCR v3.0 achieves this goal by replacing each standard probe carrying the full HCR initiator I1 (Fig. 1B, left) with a pair of cooperative split-initiator probes that each carry half of HCR initiator I1 (Fig. 1B, right). Probe pairs that hybridize specifically to their adjacent binding sites on the target mRNA colocalize the two halves of initiator I1, enabling cooperative initiation of HCR signal amplification. Meanwhile, any individual probes that bind non-specifically in the sample do not colocalize the two halves of

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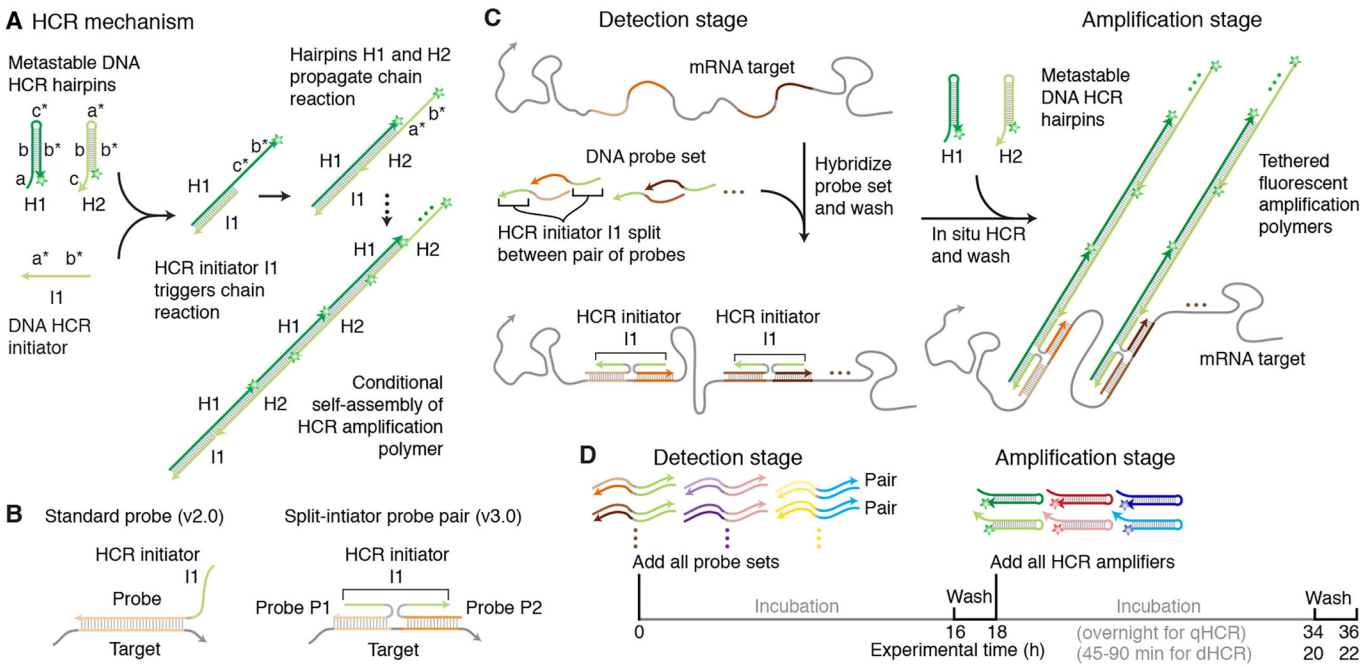

Fig. 1. *In situ* HCR v3.0 using split-initiator probes. (A) HCR mechanism. Green stars denote fluorophores. Arrowhead indicates 3′ end of each strand. (B) Standard probes carry full HCR initiator I1 and generate amplified background if they bind non-specifically. Split-initiator probes P1 and P2 each carry half of HCR initiator I1 and do not generate amplified background if they bind non-specifically. (C) Two-stage *in situ* HCR protocol. Detection stage: probe sets hybridize to mRNA targets, unused probes are washed from the sample. Amplification stage: specifically bound probe pairs trigger self-assembly of a tethered fluorescent amplification polymer and unused hairpins are washed from the sample. Automatic background suppression throughout the protocol: any reagents that bind non-specifically do not lead to generation of amplified background. (D) Multiplexing timeline. The same two-stage protocol is used independent of the number of target mRNAs. HCR amplification is performed overnight for qHCR imaging and qHCR flow cytometry experiments (to maximize the signal-to-background ratio) and for 45-90 min for dHCR imaging experiments (to resolve individual molecules as diffraction-limited dots).

initiator I1, do not trigger HCR and thus suppress generation of amplified background.

RESULTS

Validation of split-initiator HCR suppression *in vitro* and *in situ*

We first tested split-initiator HCR suppression in solution using gel studies to quantify conversion of HCR hairpins into HCR amplification polymers (Fig. 2). There is minimal leakage of hairpins H1 and H2 out of their kinetically trapped states in the absence of HCR initiator I1 (lane 1). This result demonstrates the automatic background suppression that HCR provides during the amplification stage of an *in situ* hybridization protocol: if a hairpin binds non-specifically in the sample, it does not trigger HCR and hence does not generate amplified background. As a positive control, we then verified that HCR initiator I1 triggers full conversion of HCR hairpins into amplification polymers (lane 2). If initiator I1 is carried by a standard probe, amplification polymers would represent either amplified signal or amplified background, depending on whether or not the probe is bound specifically to the

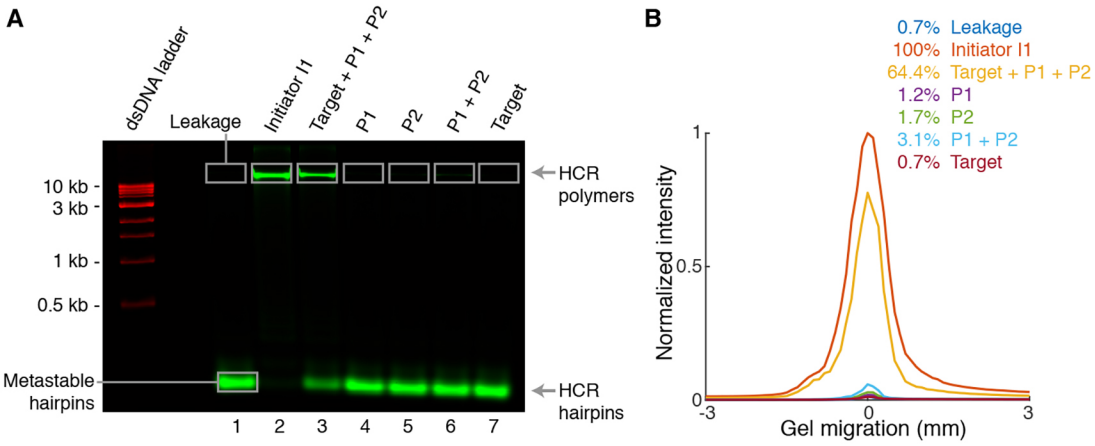

Fig. 2. Test tube validation of split-initiator HCR suppression. (A) Agarose gel electrophoresis. Reaction conditions: hairpins H1 and H2 at 0.5 μM each (lanes 1-7); initiator I1, probes P1 and P2 (each carrying half of initiator I1; Fig. 1B), and/or DNA target at 5 nM each (lanes noted on the gel); 5× SSCT buffer; overnight reaction at room temperature. Hairpins H1 and H2 were labeled with Alexa 647 fluorophore (green channel); a dsDNA 1 kb ladder was pre-stained with SYBR Gold (red channel). (B) Quantification of polymer bands in A. See Figs S3 and S4 for additional data.

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target. It is this conceptual weakness that split-initiator probes seek to eliminate. Using a pair of split-initiator probes (P1 and P2) that each carry half of HCR initiator I1, we expect HCR to be triggered if, and only if, both P1 and P2 bind specifically to their adjacent binding sites on the target. Consistent with this expectation, we observe strong conversion of hairpins H1 and H2 into amplification polymer if P1 and P2 are both introduced with the target (lane 3), but minimal conversion into polymer if either P1 or P2 is introduced alone (lanes 4 and 5), reflecting the HCR suppression capabilities of split-initiator probes. Indeed, if the target is absent, even if P1 and P2 are present in solution together, we observe minimal conversion of hairpins into polymer (lane 6). These results indicate that replacement of a standard probe (v2.0) with a pair of split-initiator probes (v3.0) is expected to modestly decrease amplified signal (lane 2 versus lane 3) but to dramatically decrease amplified background (lane 2 versus lanes 4 and 5). Gel studies of five HCR amplifiers demonstrate typical HCR suppression of ≈60-fold (Figs 2, S3 and S4, lane 3 versus lanes 4 and 5) using split-initiator probes.

We then measured split-initiator HCR suppression *in situ* by comparing the signal using full probe sets (i.e. both odd and even probes) versus partial probe sets that eliminate one probe from each pair (i.e. only odd probes or only even probes). For five HCR amplifiers, we observe typical HCR suppression of ≈50-fold (Table S9) using split-initiator probes *in situ*.

In situ validation of automatic background suppression with split-initiator probes in whole-mount chicken embryos

We next compared the performance of standard probes (v2.0) and split-initiator probes (v3.0) in whole-mount chicken embryos (Fig. 3), a representative challenging imaging setting where the sample is thick and autofluorescent. Using standard probes, as the probe set size is increased from 5 to 10 to 20 probes by adding untested probes to a previously validated set of 5 probes (Choi et al., 2016), the background increases dramatically (Fig. 3A, magenta) and the signal-to-background ratio decreases monotonically (Fig. 3B, magenta). Using split-initiator probe pairs that address nearly identical target subsequences, increasing the probe set size causes no measurable change in the background (Fig. 3A, orange) and the signal-to-background ratio increases monotonically (Fig. 3B, orange). Representative images using the largest of these unoptimized probe sets (20 standard probes or 20 split-initiator probe pairs) exhibit high background using standard probes and no visible background using split-initiator probes (Fig. 3C); corresponding pixel intensity histograms for regions of high expression (signal+background) and no or low expression (background) are overlapping using standard probes and non-overlapping using split-initiator probes (Fig. 3D). These data illustrate the significant benefit of automatic background suppression using split-initiator probes: even if there are non-specific probes in the probe set, they do not generate amplified background, so it is straightforward to increase the signal-to-background ratio simply by increasing the probe set size without probe set optimization.

This improved performance is not simply an increase in selectivity resulting from use of probes with a shorter target-binding site (50 nt for standard probes versus 25 nt for each split-initiator probe within a pair): if the split-initiator probe set with 20 probe pairs is modified so that one probe within each pair carries the full initiator I1 (with its partner carrying no initiator), the background increases by an order of magnitude (Fig. S9 and Table S12) and the signal-to-background ratio decreases by one to two orders of magnitude (Fig. S10 and Table S13).

Multiplexed mRNA imaging in whole-mount chicken embryos with large unoptimized split-initiator probe sets

To test the robustness of automatic background suppression, we performed a four-channel multiplexed experiment using large unoptimized split-initiator probe sets (v3.0) in the neural crest of whole-mount chicken embryos (Fig. 4). Three target mRNAs (*EphA4*, *Sox10*, *Dmbx1*) were each detected with 20 split-initiator probe pairs and one shorter target mRNA (*FoxD3*) was detected with 12 split-initiator probe pairs. We observed signal-to-background for each channel ranging from ~27-59 without probe

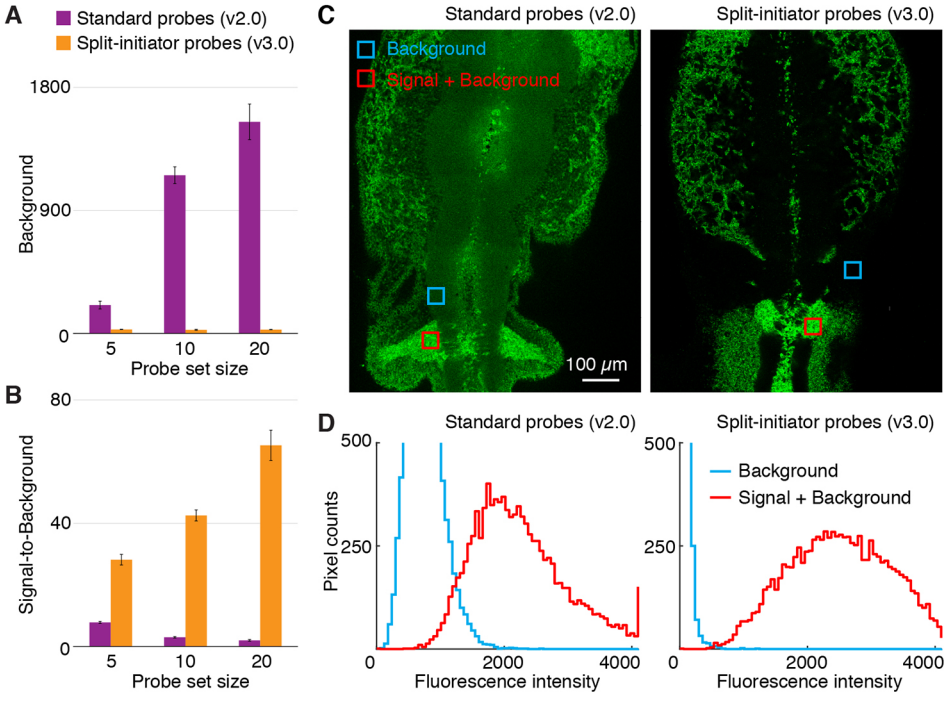

Fig. 3. *In situ* validation of automatic background suppression with split-initiator probes in whole-mount chicken embryos. (A) Fluorescent background and (B) signal-to-background ratio as probe set size is increased by adding unoptimized probes: total of 5, 10 or 20 standard probes (v2.0) versus 5, 10 or 20 split-initiator probe pairs (v3.0). Any standard probes that bind non-specifically will generate amplified background, necessitating probe set optimization; split-initiator probes eliminate the potential need for probe set optimization by providing automatic background suppression. (C) Confocal micrographs in the neural crest of fixed whole-mount chicken embryos. Unoptimized probe sets: 20 standard probes (left) or 20 split-initiator probe pairs (right). See Fig. S7 (top) for the optimized standard probe set (Choi et al., 2016) with five probes. (D) Pixel intensity histograms for background and signal plus background (pixels in the depicted regions of C): overlapping distributions using unoptimized standard probes; non-overlapping distributions using unoptimized split-initiator probes. Embryos fixed at stage HH11. Target mRNA is *Sox10*. See Figs S5-S11 and Tables S10-S14 for additional data.

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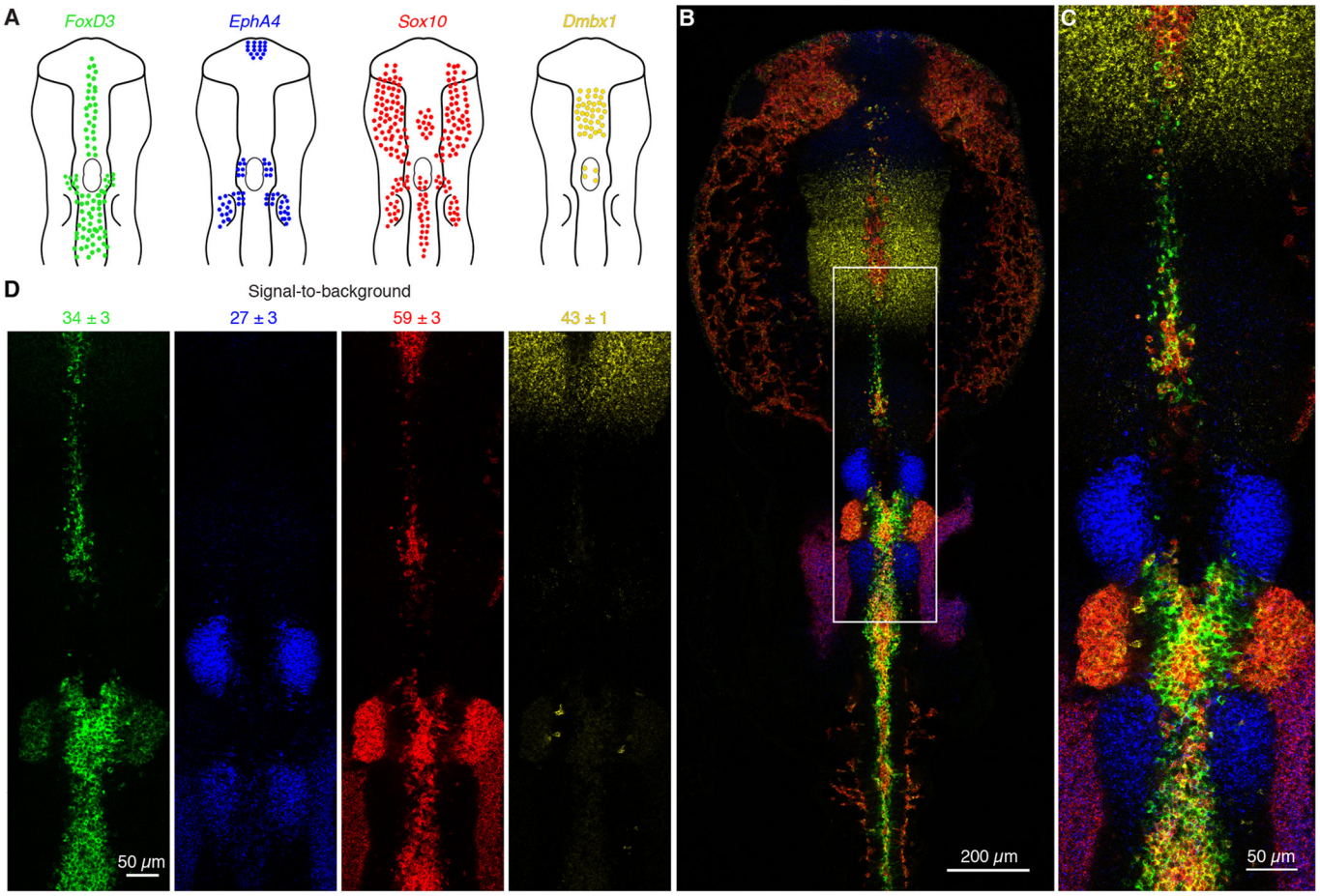

Fig. 4. Multiplexed mRNA imaging in whole-mount chicken embryos with large unoptimized probe sets using *in situ* HCR v3.0. (A) Expression schematics for four target mRNAs in the head and neural crest: *FoxD3*, *EphA4*, *Sox10* and *Dmbx1*. (B) Four-channel confocal micrograph. (C) Zoom of depicted region of B. (D) Four individual channels from C with signal-to-background ratio measurements (mean±s.e.m., *n*=3 embryos). Probe sets: 12-20 split-initiator probe pairs per target. Amplifiers: four orthogonal HCR amplifiers carrying spectrally distinct fluorophores. Embryo fixed at stage HH10. See Fig. S12 and Table S15 for additional data.

set optimization. This level of performance was achieved for all targets simultaneously in four-channel images using fluorophores that compete with lower autofluorescence (Alexa 647) as well as with higher autofluorescence (Alexa 488).

By comparison, we previously optimized standard probe sets (v2.0) for three target mRNAs (*FoxD3*, *Msx1*, *Sox10*) in the neural crest of whole-mount chicken embryos (Choi et al., 2016). Starting with 13-16 standard probes (each carrying two HCR initiators), we arrived at optimized probe sets of five to nine probes, achieving signal-to-background ratios of ∼5-8 (Choi et al., 2016). This represents good performance after an initial investment of labor to perform probe set optimization, but even optimized standard probe sets do not perform as well as unoptimized split-initiator probe sets. Split-initiator probes not only dramatically improve ease of use by removing the need for probe set optimization, they also dramatically increase the signal-to-background ratio, offering a win/win proposition over standard probes.

qHCR imaging: analog mRNA relative quantitation with subcellular resolution in an anatomical context

We have previously demonstrated that *in situ* HCR v2.0 overcomes the longstanding trade-off between RNA quantitation and anatomical context, using optimized standard HCR probe sets to perform analog mRNA relative quantitation (qHCR imaging) with subcellular resolution within whole-mount vertebrate embryos (Trivedi et al., 2018). Precision increases with probe set size (Trivedi et al., 2018), so the prospect of using large unoptimized split-initiator probe sets is highly appealing. To test mRNA relative quantitation with automatic background suppression, we redundantly detected target mRNAs using two split-initiator probe sets each triggering a different spectrally distinct HCR amplifier (Fig. 5AB). If HCR signal scales approximately linearly with the number of target mRNAs per voxel, a two-channel scatter plot of normalized voxel intensities will yield a tight linear distribution with approximately zero intercept. Conversely, observing a tight linear distribution with approximately zero intercept (Fig. 5C), we conclude that the HCR signal scales approximately linearly with the number of target mRNAs per imaging voxel, after first ruling out potential systematic crowding effects that could permit pairwise voxel intensities to slide undetected along the line (Figs S13 and S23). Using 20 unoptimized split-initiator probe pairs (v3.0) per channel, the observed accuracy (linearity with zero intercept) and precision (scatter around the line) are both excellent for subcellular 2.1×2.1×2.7 μm voxels within a whole-mount chicken embryo. Just as quantitative PCR (qPCR) enables analog mRNA relative quantitation *in vitro* (Gibson et al., 1996; Heid et al., 1996), qHCR imaging enables analog mRNA relative quantitation *in situ*.

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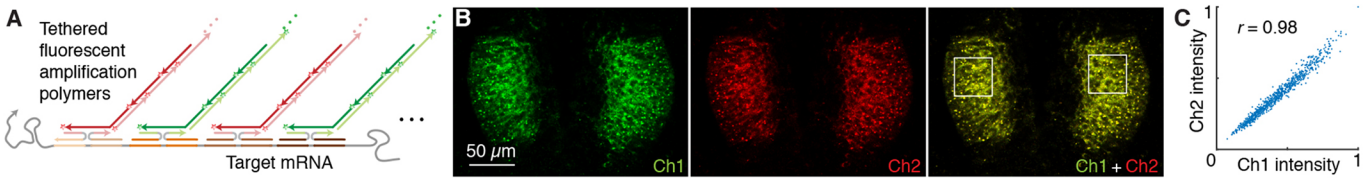

Fig. 5. qHCR imaging: analog mRNA relative quantitation with subcellular resolution in an anatomical context. (A) Two-channel redundant detection of target mRNA *EphA4* in a whole-mount chicken embryo. The target is detected using two probe sets, each initiating an orthogonal spectrally distinct HCR amplifier (Ch1, Alexa 546; Ch2, Alexa 647). (B) Confocal microscopy: 0.2×0.2 µm pixels. Probe sets: 20 split-initiator probe pairs per channel; no probe set optimization. Embryo fixed at stage HH10. (C) High accuracy and precision for mRNA relative quantitation in an anatomical context. Highly correlated normalized signal (Pearson correlation coefficient, *r*) for subcellular 2.1×2.1×2.7 µm voxels in the selected regions of B. Accuracy: linearity with zero intercept. Precision: scatter around the line. See Figs S18 and S19 and Table S16 for additional data.

qHCR flow cytometry: analog mRNA relative quantitation for high-throughput expression profiling of human and bacterial cells

The accuracy, precision and resolution achieved using qHCR imaging suggest the potential for mRNA analog relative quantitation in high-throughput flow cytometry and cell-sorting studies. In this case, the instrument treats each cell as a voxel, with both signal and background integrated over the volume of the cell. Using qHCR flow cytometry with 10-18 split-initiator probe pairs per channel (v3.0), we observe high signal-to-background (Fig. 6A) and excellent accuracy and precision (Fig. 6B) for both human and bacterial cells. Multiplexed qHCR flow cytometry (Figs S27 and S28) will enable high-throughput expression profiling without the need for engineering reporter lines (e.g. for profiling stem cell heterogeneity or sorting bacterial species in heterogeneous environmental samples).

dHCR imaging: digital mRNA absolute quantitation in an anatomical context

We have previously shown that *in situ* HCR v2.0 achieves single-molecule sensitivity and resolution even in thick autofluorescent samples (e.g. 0.5 mm cleared adult mouse brain sections) (Shah et al., 2016a), providing a basis for digital mRNA absolute quantitation (dHCR imaging). For dHCR imaging, we employ large probe sets (to distinguish mRNAs bound by multiple probes from background) and short amplification times (to grow short amplification polymers and resolve individual mRNAs as diffraction-limited dots). Because it is impractical to optimize large probe sets, it is especially appealing to use split-initiator probe sets that offer automatic background suppression and require no optimization.

To validate dHCR imaging using split-initiator probes, we redundantly detected individual mRNA targets using two independent probe sets and HCR amplifiers. We then used dot detection methods from the computer vision community to automatically identify dots in each channel (supplementary information, section S1.4.6). As mRNA false-positive and false-negative rates for each channel go to zero, the colocalization fraction for each channel (fraction of dots in a given channel that are in both channels) will approach one from below. Using large unoptimized split-initiator probe sets (23-25 split-initiator probe pairs per channel), we observe colocalization fractions of ≈0.84 in cultured human cells and whole-mount chicken embryos (Fig. 7). These results improve significantly on the colocalization fractions of ≈0.50 observed in our previous dHCR imaging studies using unoptimized standard probe sets (39 standard probes per channel) in whole-mount zebrafish embryos (Fig. S31) (Shah et al., 2016a). Just as digital PCR (dPCR) enables digital mRNA absolute quantitation *in vitro* (Vogelstein and Kinzler, 1999; Sanders et al., 2013), dHCR imaging enables digital mRNA absolute quantitation *in situ*.

DISCUSSION

Comparison of alternative probe schemes

To fully appreciate the automatic background suppression properties of split-initiator probes combined with HCR amplifiers,

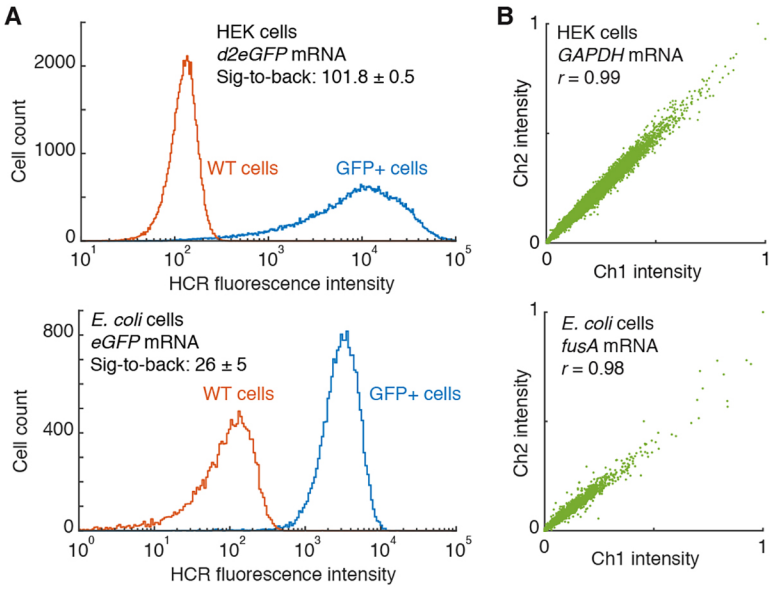

Fig. 6. qHCR flow cytometry: analog mRNA relative quantitation for high-throughput analysis of human and bacterial cells. (A) High signal-to-background ratio for transgenic target mRNAs. Data are mean±s.e.m.; *n*=55,000 HEK cells (top), *n*=18,000 *E. coli* cells (bottom). Probe sets: 12 split-initiator probe pairs; no probe set optimization. (B) High accuracy and precision for high-throughput mRNA relative quantitation. Two-channel redundant detection of endogenous target mRNAs. Each target mRNA is detected using two probe sets, each initiating an orthogonal spectrally distinct HCR amplifier (Ch1, Alexa 488; Ch2, Alexa 594). Highly correlated normalized signal (Pearson correlation coefficient, *r*), *n*=20,000 HEK cells (top), *n*=3400 *E. coli* cells (bottom). Accuracy: linearity with zero intercept. Precision: scatter around the line. Probe sets: 10 split-initiator probe pairs per channel for *GAPDH*, 18 split-initiator probe pairs per channel for *fusA*; no probe set optimization. See Figs S20-S28 and Tables S17-S24 for additional data.

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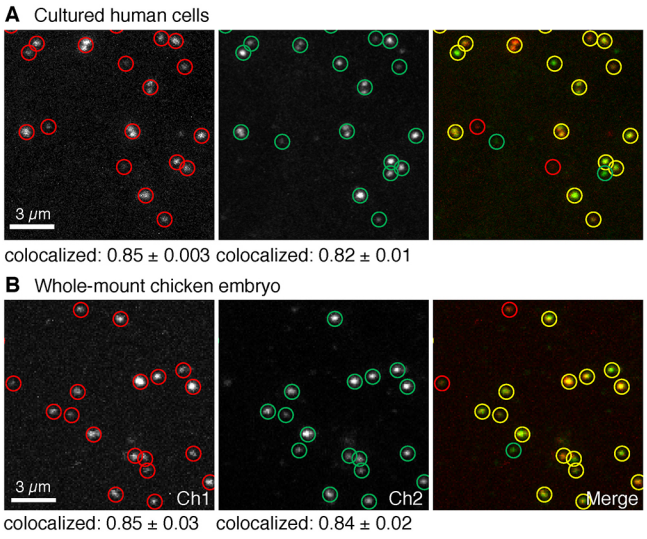

Fig. 7. dHCR imaging: digital mRNA absolute quantitation in cultured human cells and whole-mount chicken embryos. (A) Redundant detection of target mRNA *BRAF* in HEK cells. Probe sets: 23 split-initiator probe pairs per channel; no probe set optimization. Pixel size: 0.06×0.06 µm. (B) Redundant detection of target mRNA *Dmbx1* in whole-mount chicken embryos. Probe sets: 25 split-initiator probe pairs per channel; no probe set optimization. Pixel size: 0.1×0.1 µm. Embryos fixed at stage HH8. (A,B) Each target mRNA is detected using two probe sets, each initiating an orthogonal spectrally distinct HCR amplifier (Ch1, Alexa 647; Ch2, Alexa 546 for A; Ch1, Alexa 647; Ch2, Alexa 594 for B). Representative field of view from confocal micrographs. Red circles: dots detected in Ch1. Green circles: dots detected in Ch2. Yellow circles: dots detected in both channels. Colocalization represents the fraction of dots in one channel that are detected in both channels (mean±s.e.m., n=3 slides for A, n=3 embryos for B). See Figs S29-S31 and Tables S25-S27 for additional data.

it is helpful to compare alternative concepts. Fig. 8 depicts five related *in situ* hybridization schemes. In a multistage scheme, we state that a method provides automatic background suppression during a given stage if non-specific binding of a reagent during that stage predominantly does not lead to generation of amplified background during subsequent stages. As the final stage of each scheme, signal amplification is performed using HCR. Because HCR hairpins are kinetically trapped and execute a conditional self-assembly cascade that is triggered by the HCR initiator, hairpins that bind non-specifically within the sample predominantly do not trigger growth of HCR amplification polymers. Hence, HCR provides automatic background suppression during the final stage of all five schemes. The challenge, then, is to devise a probe concept that maintains automatic background suppression during the earlier stages of the protocol.

To provide a starting point for discussion, scheme A depicts the standard probes used for *in situ* HCR v2.0 (Choi et al., 2014, 2016; Shah et al., 2016a; Trivedi et al., 2018). As previously noted, because each probe carries an exposed HCR initiator I1, this scheme has the drawback that non-specific probe binding in stage 1 will lead to generation of amplified background during stage 2.

Scheme B resolves this issue by using a hairpin probe that sequesters HCR initiator I1, exposing the initiator only upon hybridization to the target. As a result, probes that bind non-specifically during stage 1 predominantly do not generate amplified background during stage 2, ensuring automatic background suppression throughout the protocol. Unfortunately, suppressing background via conformation change of a hairpin probe imposes sequence dependence between the target and the HCR amplifier, which would necessitate use of a custom HCR amplifier for each new target.

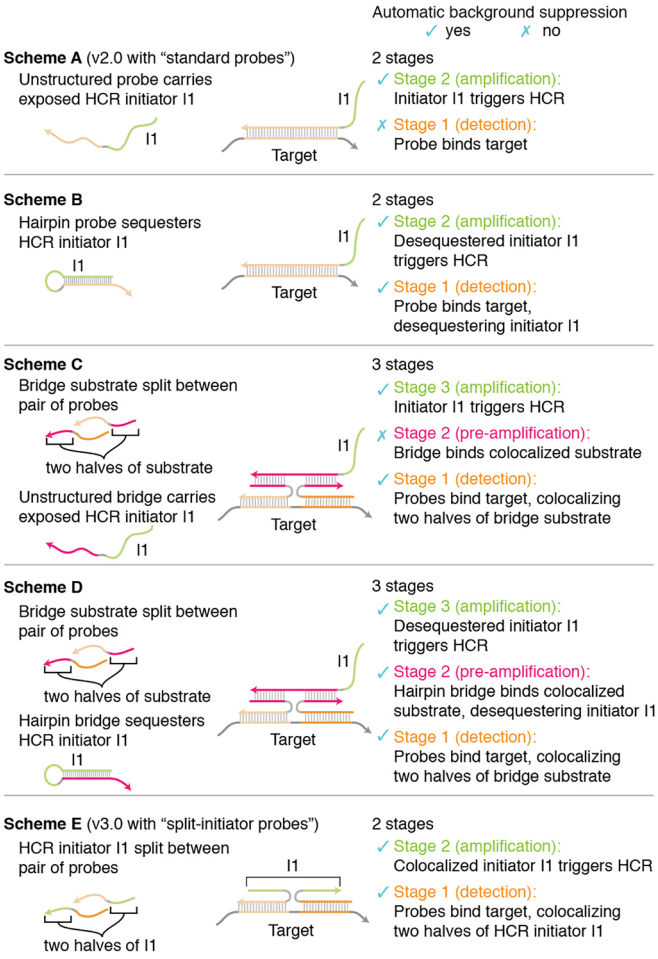

Fig. 8. Comparison of probe concepts. Scheme A corresponds to *in situ* HCR v2.0 with standard probes. Scheme E corresponds to *in situ* HCR v3.0 with split-initiator probes. Scheme A is vulnerable to non-specific probe binding in stage 1, leading to amplified background in stage 2. Scheme B provides automatic background suppression throughout the protocol at the cost of introducing sequence dependence between the target and the HCR amplifier. Scheme C provides automatic background suppression in stage 1 but is vulnerable to non-specific bridge binding in stage 2, leading to amplified background in stage 3 [a weakness shared by the pre-amplification and amplification stages (stages 2 and 3) of four-stage bDNA methods (Wang et al., 2012)]. Scheme D provides automatic background suppression throughout the protocol at the cost of using a three-stage protocol. Scheme E offers all of the advantages and none of the disadvantages of schemes A-D, providing automatic background suppression throughout the protocol, avoiding sequence dependence between the HCR amplifier and the target mRNA, and employing a two-stage protocol. Arrowhead indicates the 3′ end of each strand.

To sidestep this sequence-dependence issue, scheme C uses colocalization instead of conformation change as an alternative principle for achieving automatic background suppression. During stage 1, the target is detected using a pair of probes that each carry half of a bridge substrate. Specific hybridization of the probes to the target molecule colocalizes the two halves of the bridge substrate. During stage 2, an unstructured bridge strand that carries exposed HCR initiator I1 is designed to bind stably to the colocalized substrate, but not to either half alone. Thus, non-specific binding of either probe during stage 1 predominantly will not generate amplified background during stage 2. The drawback to scheme C is that non-specific binding of the bridge strand during stage 2 will lead to generation of amplified background during stage 3. In

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essence, the unstructured bridge strand in scheme C has the same conceptual weakness as the unstructured probe in scheme A.

The principles, strengths and weaknesses underlying stages 1 and 2 of scheme C are similar to those of branched DNA methods (bDNA), which use a four-stage protocol (Wang et al., 2012): stage 1, target detection with a pair of probes each carrying half of a bridge substrate; stage 2, pre-amplification with an unstructured bridge strand that binds to a colocalized bridge substrate and carries multiple exposed amplifier substrates; stage 3, amplification with an unstructured amplifier strand that binds to an exposed amplifier substrate and carries multiple exposed label substrates; stage 4, signal generation with an unstructured label strand that binds to an exposed label substrate. This approach has the conceptual strength that non-specific binding of individual probes during stage 1 will predominantly not lead to generation of amplified background (as only bridge substrates colocalized by the target will mediate amplification), but also the conceptual weakness that non-specific binding of reagents in stages 2 or 3 will lead to generation of amplified background (as unstructured bridge strands carry exposed amplifier substrates and unstructured amplifier strands carry exposed label substrates). Hence, automatic background suppression is achieved in stage 1 based on the principle of colocalization, but then not maintained during stages 2 and 3 as a result of reliance on unstructured strands that carry exposed substrates for downstream reagents.

To achieve automatic background suppression throughout the protocol, scheme D improves on scheme C by replacing the unstructured bridge strand with a hairpin bridge that initially sequesters HCR initiator I1, exposing I1 only upon hybridizing to the colocalized bridge substrate. Automatic background suppression is achieved in stage 1 based on the principle of colocalization and then maintained during stage 2 based on the principle of conformation change. The drawback of scheme D is the increase in number of stages from 2 to 3.

As the final step in the derivation of split-initiator probes, scheme E simplifies scheme D by noting that the conformation-change property of the hairpin bridge is also a property of the HCR hairpins used for amplification. Therefore, with scheme E, we stipulate that the bridge substrate is an HCR initiator sequence, enabling HCR hairpins to bridge between colocalized probes and amplify the signal in a single stage. As a result, scheme E becomes a two-stage protocol.

Scheme E, which provides the basis for *in situ* HCR v3.0 in the current work, provides all of the benefits and none of the drawbacks of the other four schemes. First, we have the simplicity of a two-stage protocol (stage 1, detection; stage 2, amplification). Second, we have the flexibility of sequence independence between the target and the HCR amplifier, enabling use of a validated library of HCR amplifiers for new targets of interest. Third, we have the robustness of automatic background suppression throughout the protocol: at every stage during the protocol, non-specific binding of reagents will predominantly not lead to generation of amplified background.

Enhanced robustness and signal-to-background

Automatic background suppression using split-initiator probes has important consequences for both robustness and signal-to-background. Using standard probes, increasing the size of the probe set will reliably increase amplified signal but might increase amplified background even more, so use of a large v2.0 probe set can be a double-edged sword; probe set optimization is sometimes required to ensure that increasing probe set size does more good than harm. By contrast, using split-initiator probe sets, the signal-to-background ratio increases reliably with probe set size, so it is advantageous to use large v3.0 probe sets without optimization and achieve a high signal-to-background ratio on the first try. Even compared with optimized v2.0 probe sets, unoptimized v3.0 probe sets lead to a dramatically higher signal-to-background ratio, so performance improves even though we dispense with the extra effort of probe set optimization.

qHCR and dHCR quantitative imaging modes

In situ HCR enables two quantitative imaging modes in thick autofluorescent samples:

(1) qHCR imaging – analog mRNA relative quantitation with subcellular resolution; HCR signal is analog in the form of fluorescence voxel intensities that scale approximately linearly with the number of target molecules per voxel.

(2) dHCR imaging – digital mRNA absolute quantitation; HCR signal is digital in the form of diffraction-limited dots representing individual target molecules.

For qHCR imaging, we recommend using 20 split-initiator probe pairs per target and amplifying overnight. For dHCR imaging, we recommend maximizing the number of probe pairs per target (at least 25 probe pairs is preferred) and amplifying for 45-90 min. Because the qHCR signal per imaging voxel is quantitative, it will naturally decrease to zero as the number of targets per voxel decreases to zero; for sufficiently low expression, the signal will not be observable above autofluorescence. However, the dHCR signal per target molecule does not decrease with expression level. Hence, the qHCR and dHCR quantitative imaging modes are complementary, with qHCR suitable for medium- and high-copy targets (where the quantitative signal dominates autofluorescent background), and dHCR suitable for low-copy targets (where the signal from individual target molecules can be spatially separated). The same probe set can be used for either imaging mode, so imaging can be performed in qHCR mode (longer amplification time, lower magnification) or dHCR imaging mode (shorter amplification time, higher magnification) depending on the expression level observed *in situ*.

Quantitative read-out and read-in

The quantitative properties of *in situ* HCR enable gene expression queries in two directions (Trivedi et al., 2018): read-out from anatomical space to expression space to discover co-expression relationships in selected regions of the specimen; conversely, read-in from multidimensional expression space to anatomical space to discover those anatomical locations in which selected gene co-expression relationships occur. Quantitative read-out and read-in analyses provide the strengths of flow cytometry expression analyses, but by preserving anatomical context, they enable bi-directional queries that open a new era for *in situ* hybridization (Trivedi et al., 2018). *In situ* HCR v3.0 using large split-initiator probe sets enhances accuracy and precision for read-out/read-in using either qHCR relative quantitation (Trivedi et al., 2018) or dHCR absolute quantitation (Shah et al., 2016b).

In situ HCR resolves longstanding shortcomings of traditional CARD *in situ* amplification methods

Fluorescent *in situ* hybridization methods are used across the life sciences to image mRNA expression within fixed cells, tissues and organisms. In challenging imaging settings, including whole-mount vertebrate embryos and thick tissue sections, autofluorescence within the sample necessitates the use of *in situ* amplification to boost the signal-to-background ratio (Tautz and Pfeifle, 1989; Harland, 1991; Lehmann and Tautz, 1994; Kerstens et al., 1995; Nieto et al., 1996;

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Wiedorn et al., 1999; Player et al., 2001; Pernthaler et al., 2002; Denkers et al., 2004; Kosman et al., 2004; Larsson et al., 2004, 2010; Thisse et al., 2004; Zhou et al., 2004; Clay and Ramakrishnan, 2005; Barroso-Chinea et al., 2007; Acloque et al., 2008; Piette et al., 2008; Thisse and Thisse, 2008; Weiszmann et al., 2009; Wang et al., 2012). For decades, traditional *in situ* amplification approaches based on catalytic reporter deposition (CARD) have been the dominant approach for generating high signal-to-background in samples with high autofluorescence (Tautz and Pfeifle, 1989; Harland, 1991; Lehmann and Tautz, 1994; Kerstens et al., 1995; Nieto et al., 1996; Pernthaler et al., 2002; Denkers et al., 2004; Kosman et al., 2004; Thisse et al., 2004; Clay and Ramakrishnan, 2005; Barroso-Chinea et al., 2007; Acloque et al., 2008; Piette et al., 2008; Thisse and Thisse, 2008; Weiszmann et al., 2009) despite three significant drawbacks: multiplexing is cumbersome due to the need to perform signal amplification for one target mRNA at a time (Lehmann and Tautz, 1994; Nieto et al., 1996; Denkers et al., 2004; Kosman et al., 2004; Thisse et al., 2004; Clay and Ramakrishnan, 2005; Barroso-Chinea et al., 2007; Acloque et al., 2008; Piette et al., 2008); staining is qualitative rather than quantitative; and spatial resolution is routinely compromised by diffusion of reporter molecules prior to deposition (Tautz and Pfeifle, 1989; Thisse et al., 2004; Acloque et al., 2008; Piette et al., 2008; Thisse and Thisse, 2008; Weiszmann et al., 2009).

In situ HCR v2.0 overcame these longstanding difficulties, enabling multiplexed, quantitative, high-resolution imaging of mRNA expression with high signal-to-background in diverse organisms, including whole-mount vertebrate embryos (Choi et al., 2014, 2016; Trivedi et al., 2018). Orthogonal HCR amplifiers operate independently within the sample so the experimental timeline for multiplexed experiments is independent of the number of target mRNAs (Choi et al., 2010, 2014). The amplified HCR signal scales approximately linearly with the number of target molecules, enabling accurate and precise mRNA relative quantitation with subcellular resolution in the anatomical context of whole-mount vertebrate embryos (Trivedi et al., 2018). Amplification polymers remain tethered to their initiating probes, enabling imaging of mRNA expression with subcellular or single-molecule resolution as desired (Choi et al., 2014, 2016; Shah et al., 2016a). With split-initiator probes, *in situ* HCR v3.0 adds the performance and robustness benefits of automatic background suppression, providing biologists with an enhanced state-of-the-art research tool for the study of mRNA expression (Table 1).

MATERIALS AND METHODS

Probe sets, amplifiers and buffers

For each target mRNA, a kit containing a DNA probe set, a DNA HCR amplifier, and hybridization, wash and amplification buffers was purchased from Molecular Technologies (moleculartechnologies.org), a non-profit academic resource within the Beckman Institute at Caltech. For gel studies, see Table S1 for sequence information. For *in situ* HCR studies, see Table S2 for a summary of sample, probe set and amplifier details, and Section S4 for probe sequences. Sequences for HCR amplifiers B1, B2, B3, B4 and B5 are given in Choi et al. (2014).

Table 1. mRNA imaging using *in situ* HCR

Property	Details
Simple	Two-stage protocol independent of number of targets
Amplified	Boosts signal above autofluorescence
Multiplexed	Simultaneous one-stage amplification for up to five targets
Quantitative	Signal scales linearly with target abundance
Penetrating	Whole-mount vertebrate embryos and thick tissue sections
Resolved	Subcellular or single-molecule resolution as desired
Sensitive	Single molecules detected in thick autofluorescent samples
Versatile	Suitable for use with diverse targets in diverse organisms
Robust	Automatic background suppression throughout protocol

Gel electrophoresis

DNA HCR reactions for Fig. 2, Figs S3 and S4 were performed in 5× SSCT (5× SSC with 0.1% Tween 20). All hairpins were labeled with Alexa 647 (green channel). DNA hairpins were snap-cooled separately at 3 µM in hairpin storage buffer (Molecular Technologies). DNA initiators (I1), split-initiator probes (P1, P2), and target (Target) were diluted to 0.03 µM in 5× SSC. Each lane was prepared by mixing 0.8 µl of 5× SSC and 1.2 µl of 5× SSC with 1% Tween 20 and 2 µl of each hairpin. For the lanes with DNA oligos (I1, P1, P2 or Target), 2 µl of each oligo was added. An appropriate amount of 5× SSC was added to each lane to bring the reaction volume to 12 µl. The reactions were incubated at room temperature overnight. The samples were supplemented with 3 µl of 5× gel loading buffer (50% glycerol with Bromophenol Blue and xylene cyanol tracking dyes) and loaded into a native 1% agarose gel, prepared with 1× LB buffer (Faster Better Media). The gel was run at 150 V for 60 min at room temperature and imaged using an FLA-5100 fluorescent scanner (Fujifilm Life Science) with a 635 nm laser and a 665 nm long-pass filter. The 1 kb DNA ladder (red channel) was prestained with SYBR Gold (Invitrogen) and imaged using a 488 nm laser and a 575 nm long-pass filter. Multi Gauge software (Fuji Photo Film) was used to calculate the Alexa 647 intensity profile surrounding the polymer band for each lane (lanes 1-7 in Fig. 2, Figs S3, and S4). Each intensity profile is displayed for ±3 mm of gel migration distance with the peak value centered at 0; the intensity values are normalized so that the highest peak value for each gel is set to 1. Signal for each band was calculated using Multi Gauge with auto-detection of signal and background; the calculated percentages were normalized to the measured value with the full initiator (lane 2). Based on repeated analysis using Multi Gauge, the uncertainty in quantifying the bands in any given gel is estimated to be less than 0.1% of the band signal used for normalization.

In situ hybridization

In situ HCR v2.0 with standard probes was performed using the whole-mount chicken protocols of Choi et al. (2016). *In situ* HCR v3.0 with split-initiator probes was performed using the protocols detailed in section S2 of the supplementary material. Experiments were performed in *Gallus gallus domesticus* embryos (fertilized white leghorn chicken eggs from McIntyre Poultry & Fertile Eggs; fixed HH8, HH10 or HH11), human embryonic kidney (HEK) 293T cells (ATCC, # CRL-3216), HEK293 d2eGFP cells (a gift from C. L. Beisel, Caltech, Pasadena, CA, USA), *E. coli* K12 MG1655 (a gift from A. Z. Rosenthal, Caltech, Pasadena, CA, USA) or *E. coli* K12 MG1655 pUA66-sdhC expressing *gfpmut2* (a gift from A. Z. Rosenthal). For signal amplification in analog qHCR mode (a high signal-to-background ratio with quantitative voxel intensities for imaging with subcellular resolution or high-throughput flow cytometry; e.g. Figs 3-6), amplification was performed overnight to generate long HCR amplification polymers. For signal amplification in digital dHCR mode (single-molecule sensitivity and resolution with individual target molecules resolved as diffraction-limited dots; e.g. Fig. 7), amplification was performed for 45-90 min to generate short HCR amplification polymers. See Table S2 for the amplification time for each experiment.

Confocal microscopy

A Zeiss LSM 710 inverted confocal microscope equipped with an LD C-Apochromat 40×/1.1 W Korr M27 objective was used to image whole-mount chicken embryos in Figs 3 and 5, Figs S5-S11,S14-S19. The same microscope equipped with an LD LCI Plan-Apochromat 25×/0.8 Imm Korr DIC M27 was used to image four-color whole-mount chicken embryos in Fig. 4 and Fig. S12. A Zeiss LSM 800 inverted confocal microscope equipped with a Plan-Apochromat 63×/1.4 Oil DIC M27 objective was used to image whole-mount chicken embryos in Fig. 7 and Fig. S30. The same microscope equipped with an alpha Plan-Apochromat 100×/1.46 Oil DIC (UV) M27 objective was used to image mammalian cells in Fig. 7 and

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Fig. S29. See Table S3 for a summary of excitation laser sources, beam splitters and tuned emission bandpass filters used for each experiment. All images are displayed without background subtraction. For dHCR imaging studies (e.g. Fig. 7), TetraSpeck Microspheres (0.2 µm, fluorescent blue/green/orange/dark red; Thermo Fisher Scientific, Cat. # T7280) were used as references for channel alignment. Images from two channels were registered using the Channel Alignment feature in ZEN Black software (Zeiss) and registration parameters were recorded for alignment of data imaged in dHCR 2-channel redundant detection experiments using identical imaging settings.

Flow cytometry

Prior to flow cytometry, cells were filtered through a 35 µm or a 40 µm mesh. Flow cytometry studies were performed using a MACSQuant VYB (Miltenyi Biotec). See Table S4 for a summary of excitation laser sources and filters used for each experiment. Flow cytometry data were gated using EasyFlow (Antebi et al., 2017) and plotted using MATLAB (Mathworks). For HEK cells, two gates were applied to data (e.g. Fig. S1): a first gate of forward scatter area (FSC-A) versus side scatter area (SSC-A) to select cells, and a second gate of FSC-A versus forward scatter height (FSC-H) to select single cells. Only cells satisfying both gates were used for the analysis. For *E. coli* cells, one gate of FSC-A versus SSC-A was applied to select cells (e.g. Fig. S2).

Image analysis

Image analysis was performed as detailed in section S1.4 of the supplementary material, including: definition of raw pixel intensities, measurement of signal, background and signal-to-background ratio, measurement of background components, measurement of split-initiator HCR suppression, calculation of normalized subcellular voxel intensities for qHCR imaging, and dot detection and colocalization for dHCR imaging (a dot detection and colocalization script is available for download at www.moleculartechnologies.org, including a user guide, sample images for testing the script, and sample output files).

Flow cytometry data analysis

Flow cytometry data analysis was performed as detailed in section S1.5 of the supplementary material, including: definition of raw cell intensities, measurement of signal, background and signal-to-background ratio, measurement of background components, measurement of split-initiator HCR suppression, and calculation of normalized single-cell intensities for qHCR flow cytometry.

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Competing interests

The authors declare competing financial interests in the form of patents, pending patent applications and a startup company (Molecular Instruments).

Author contributions

Conceptualization: H.M.T.C., N.A.P.; Methodology: H.M.T.C., M.S., M.E.F., N.A.P.; Software: M.E.F., J.S., A.C.; Validation: H.M.T.C., M.S.; Investigation: H.M.T.C., M.S., A.A., G.A.; Writing - original draft: H.M.T.C., M.S., N.A.P.; Writing - review & editing: H.M.T.C., M.S., M.E.F., A.A., G.A., J.S., A.C., N.P.; Visualization: H.M.T.C., M.S., N.A.P.; Supervision: N.A.P.; Project administration: N.A.P.; Funding acquisition: N.A.P.

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Supplementary information

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Supplementary Information

Third-generation in situ hybridization chain reaction: multiplexed, quantitative, sensitive, versatile, robust

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List of Figures

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S1 Additional materials and methods

S1.1 Probe set and amplifier details

HCR Amplifier	Oligo	Length (nt)	Sequence (5′ to 3′)	Figures
B1-Alexa647	I1	36	gAggAgggCAgCAAACgggAAgAgTCTTCCTTTACg	S3
	P1	45	gAggAgggCAgCAAACggAAAgACgTTgTggCTgTTgTAgTTgTA	S3
	P2	45	CgTTCTTCTgCTTgTCggCCATgATTAgAAgAgTCTTCCTTTACg	S3
B2-Alexa647	I1	36	CCTCgTAAATCCTCATCAATCATCCAgTAAACCgCC	S3
	P1	45	CCTCgTAAATCCTCATCAAAAgACgTTgTggCTgTTgTAgTTgTA	S3
	P2	45	CgTTCTTCTgCTTgTCggCCATgATAAATCATCCAgTAAACCgCC	S3
B3-Alexa647	I1	36	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	2, S4
	P1	45	gTCCCTgCCTCTATATCTTTAgACgTTgTggCTgTTgTAgTTgTA	2, S4
	P2	45	CgTTCTTCTgCTTgTCggCCATgATTTCCACTCAACTTTAACCCg	2, S4
B4-Alexa647	I1	36	CCTCAACCTACCTCCAACTCTCACCATATTCgCTTC	S3
	P1	45	CCTCAACCTACCTCCAACAAAgACgTTgTggCTgTTgTAgTTgTA	S3
	P2	45	CgTTCTTCTgCTTgTCggCCATgATATTCTCACCATATTCgCTTC	S3
B5-Alexa647	I1	36	CTCACTCCCAATCTCTATCTACCCTACAAATCCAAT	S3
	P1	45	CTCACTCCCAATCTCTATAAAgACgTTgTggCTgTTgTAgTTgTA	S3
	P2	45	CgTTCTTCTgCTTgTCggCCATgATAACTACCCTACAAATCCAAT	S3

Table S1. Sequences for gel studies (cf. Figure 2). Initiator sequence in green, spacer sequence in blue, target-binding sequence in black. In all cases, the Target is: 5′-TACAACTACAACAgCCACAACgTCTATATCATggCCgACAAgCAgAAgAACg-3′.

Organism	Target	Standard probes (v2.0)	Split-initiator probe pairs (v3.0)	HCR amplifier	Amplification time	Figures
G. gallus domesticus	*Sox10*	5, 10, 20		B3-Alexa647	overnight	3, S5, S7
	Sox10		5, 10, 20	B3-Alexa647	overnight	3, S6, S8
	Sox10		20	B3-Alexa647	overnight	S9, S10
	Sox10	20		B3-Alexa647	overnight	S9, S10
	EphA4		20	B2-Alexa647	overnight	S11
	FoxD3		12	B4-Alexa488	overnight	4, S12
	Dmbx1		20	B1-Alexa514	overnight	4, S12
	Sox10		20	B3-Alexa546	overnight	4, S12
	EphA4		20	B2-Alexa647	overnight	4, S12
	EphA4		20	B1-Alexa546	overnight	S13, S14, S15
	Egr2		20	B3-Alexa647	overnight	S13, S14, S16
	Dmbx1		20	B1-Alexa546	overnight	S18
	Dmbx1		20	B2-Alexa647	overnight	S18
	EphA4		20	B1-Alexa546	overnight	5, S19
	EphA4		20	B2-Alexa647	overnight	5, S19
H. sapiens sapiens	*d2eGFP*		12	B3-Alexa594	overnight	6A, S20
	GAPDH		10	B5-Alexa488	overnight	6B, S23, S24
	GAPDH		10	B4-Alexa594	overnight	6B, S1, S21, S24
	ACTB		10	B2-Alexa594	overnight	S23
	PGK1		18	B1-Alexa488	overnight	S25
	PGK1		18	B2-Alexa594	overnight	S25, S27
	GAPDH		10	B4-Alexa488	overnight	S27
E. coli	*eGFP*		12	B3-Alexa594	overnight	6A, S22
	fusA		18	B3-Alexa488	overnight	6B, S26, S28
	fusA		18	B2-Alexa594	overnight	6B, S2, S26
	icd		20	B1-Alexa594	overnight	S28
H. sapiens sapiens	*BRAF*		23	B3-Alexa647	45 min	7A, S29
	BRAF		23	B4-Alexa546	45 min	7A, S29
G. gallus domesticus	*Dmbx1*		25	B1-Alexa594	90 min	7B, S30
	Dmbx1		25	B2-Alexa647	90 min	7B, S30

Table S2. Organisms, target mRNAs, probe sets, amplifiers, and figure numbers for in situ HCR experiments.

S1.2 Confocal microscope settings

Target	Fluorophore	Laser (nm)	Beam Splitter	Filter (nm)	Pixel size ($x \times y \times z$ μm)	Voxel size ($x \times y \times z$ μm)	Focal planes	Figures
EphA4	Alexa647	633	MBS 488/561/633	650–689	$0.208 \times 0.208 \times 2.7$		1	S11
Sox10	Alexa647	633	MBS 488/561/633	650–699	$0.415 \times 0.415 \times 2.7$		1	3, S5–S10
FoxD3	Alexa488	488	MBS 488/561/633	491–525	$0.664 \times 0.664 \times 4$		1	4, S12
Dmbx1	Alexa514	514	MBS 458/514	546–564	$0.664 \times 0.664 \times 4$		1	4, S12
Sox10	Alexa546	561	MBS 488/561/633	573–612	$0.664 \times 0.664 \times 4$		1	4, S12
EphA4	Alexa647	633	MBS 488/561/633	654–687	$0.664 \times 0.664 \times 4$		1	4, S12
Dmbx1	Alexa546	561	MBS 488/561/633	563–592	$0.208 \times 0.208 \times 2.7$	$2.1 \times 2.1 \times 2.7$	1	5, S18
Dmbx1	Alexa647	633	MBS 488/561/633	650–689	$0.208 \times 0.208 \times 2.7$	$2.1 \times 2.1 \times 2.7$	1	5, S18
EphA4	Alexa546	561	MBS 488/561/633	563–592	$0.208 \times 0.208 \times 2.7$	$2.1 \times 2.1 \times 2.7$	1	5, S19
EphA4	Alexa647	633	MBS 488/561/633	650–689	$0.208 \times 0.208 \times 2.7$	$2.1 \times 2.1 \times 2.7$	1	5, S19
BRAF	Alexa546	561	MBS 405/488/561/640 (T10/R90)	566–623	$0.0624 \times 0.0624 \times 0.42$		17	7A, S29
BRAF	Alexa647	640	MBS 405/488/561/640 (T10/R90)	656–700	$0.0624 \times 0.0624 \times 0.42$		17	7A, S29
—	DAPI	405	MBS 405/488/561/640 (T10/R90)	410–470	$0.0624 \times 0.0624 \times 0.42$		17	S29
Dmbx1	Alexa594	561	MBS 405/488/561/640 (T10/R90)	580–647	$0.099 \times 0.099 \times 0.420$		22	7B, S30
Dmbx1	Alexa647	640	MBS 405/488/561/640 (T10/R90)	645–700	$0.099 \times 0.099 \times 0.420$		22	7B, S30
EphA4	Alexa546	561	MBS 488/561/633	563–592	$0.415 \times 0.415 \times 2.7$	$2.1 \times 2.1 \times 2.7$	1	S14–S17
Egr2	Alexa647	633	MBS 488/561/633	650–689	$0.415 \times 0.415 \times 2.7$	$2.1 \times 2.1 \times 2.7$	1	S14–S17

Table S3. Confocal microscope settings.

S1.3 Flow cytometer settings

Target	Fluorophore	Laser (nm)	Filter	Emission (nm)	Figures
d2eGFP	Alexa594	561	Y2	615/20	6A, S20
GAPDH	Alexa488	488	B1	525/50	6B, S23, S24, S27
GAPDH	Alexa594	561	Y2	615/20	6B, S1, S21, S24
ACTB	Alexa594	561	Y2	615/20	S23
PGK1	Alexa488	488	B1	525/50	S25
PGK1	Alexa594	561	Y2	615/20	S25, S27
eGFP	Alexa594	561	Y2	615/20	6A, S22
fusA	Alexa488	488	B1	525/50	6B, S26, S28
fusA	Alexa594	561	Y2	615/20	6B, S2, S26
icd	Alexa594	561	Y2	615/20	S28

Table S4. Flow cytometer settings.

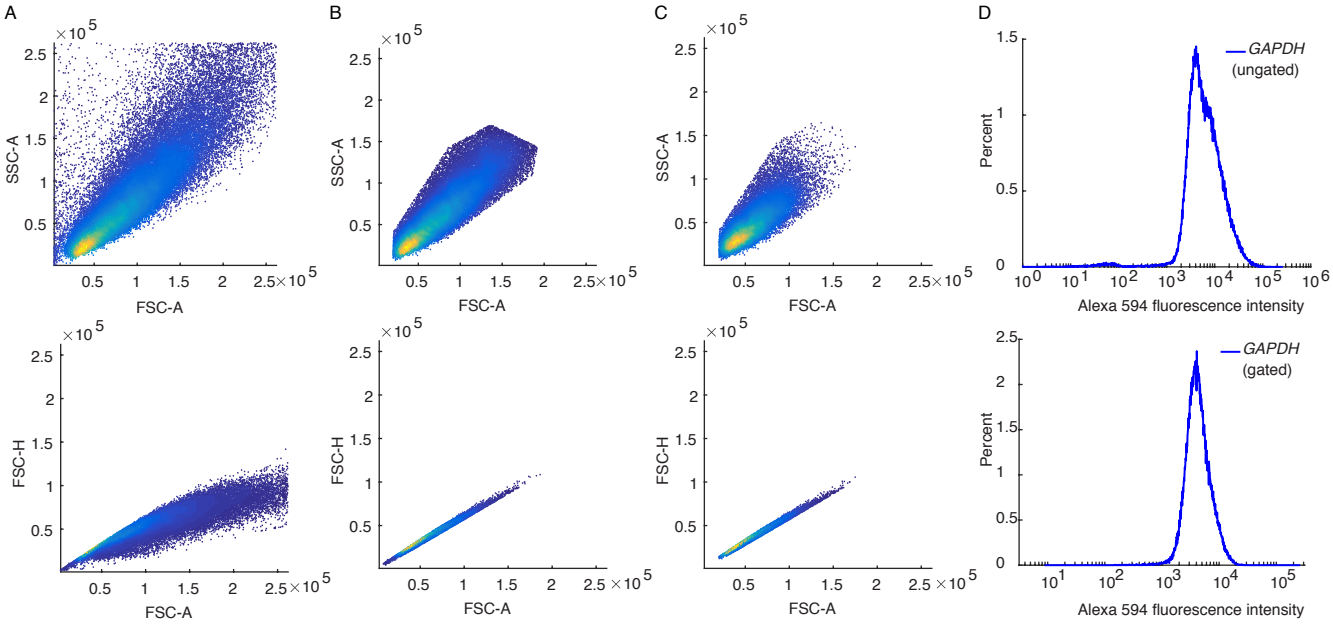

Figure S1. Illustration of gates used for flow cytometry analysis of HEK cells. (A) Scatter plots for ungated data. Top: side scatter area (SSC-A) vs forward scatter area (FSC-A). Bottom: forward scatter height (FSC-H) vs forward scatter area (FSC-A). (B) Scatter plots after applying one gate. Top: gate on FSC-A vs SSC-A to remove debris and select cells. Bottom: gate on FSC-A vs FSC-H to remove clumps of cells and select single cells. (C) Scatter plots after applying both gates. (D) Signal plus background for ungated (top) and gated (bottom) samples. Target: *GAPDH*. Probe set: 10 split-initiator probe pairs. Amplifier: B4-Alexa594. The depicted gates were used for the SIG+BACK data in Figure S21.

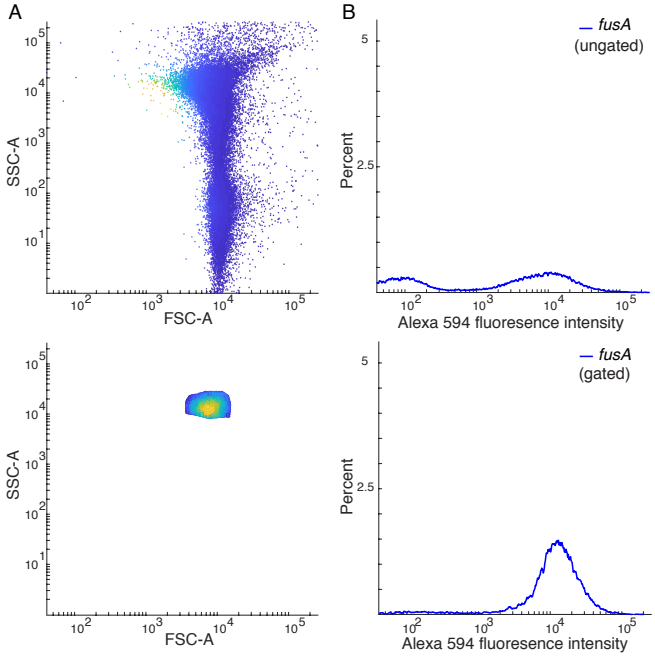

Figure S2. Illustration of gates used for flow cytometry analysis of *E. coli*. (A) Scatter plots for ungated sample (top) and gated sample (bottom): side scatter area (SSC-A) vs forward scatter area (FSC-A). (B) Signal plus background for ungated sample (top) and gated sample (bottom). Target: *fusA*. Probe set: 18 split-initiator probe pairs. Amplifier: B2-Alexa594. The depicted gate was used for the SIG+BACK data in Figure S26.

S1.4 Image analysis

We build on an image analysis framework developed over a series of publications (Choi *et al.*, 2010, 2014, 2016; Trivedi *et al.*, 2018). For convenience, here we provide a self-contained description of the details relevant to the present work.

S1.4.1 Raw pixel intensities

The total fluorescence within a pixel is a combination of signal and background. Fluorescent background (BACK) arises from three sources in each channel:

- autofluorescence (AF): fluorescence inherent to the sample,
- non-specific detection (NSD): probes that bind non-specifically in the sample and subsequently trigger HCR amplification,
- non-specific amplification (NSA): HCR hairpins that bind non-specifically in the sample.

Fluorescent signal (SIG) in each channel corresponds to:

- signal (SIG): probes that hybridize specifically to the target mRNA and subsequently trigger HCR amplification.

For pixel i of replicate embryo n, we denote the background

$$X_{n,i}^{\mathrm{BACK}} = X_{n,i}^{\mathrm{NSD}} + X_{n,i}^{\mathrm{NSA}} + X_{n,i}^{\mathrm{AF}}, \tag{S1}$$

the signal:

$$X_{n,i}^{\mathrm{SIG}}, \tag{S2}$$

and the total fluorescence (SIG+BACK):

$$X_{n,i}^{\mathrm{SIG+BACK}} = X_{n,i}^{\mathrm{SIG}} + X_{n,i}^{\mathrm{BACK}}. \tag{S3}$$

S1.4.2 Measurement of signal, background, and signal-to-background

For each target mRNA, background (BACK) is characterized for pixels in a representative rectangular region of no- or low-expression and the combination of signal plus background (SIG+BACK) is characterized for pixels in a representative rectangular region of high expression (e.g., Figures 3C, S7A, S8A, S10A, and S12A). For the pixels in these regions, we characterize the distribution by plotting an intensity histogram (e.g., Figures 3D, S7B, S8B, S10B, and S12B) and characterize average performance by calculating the mean pixel intensity ($\bar{X}_n^{\mathrm{BACK}}$ or $\bar{X}_n^{\mathrm{SIG+BACK}}$ for replicate embryo n). Performance across replicate embryos is characterized by calculating the sample means ($\bar{X}^{\mathrm{BACK}}$ and $\bar{X}^{\mathrm{SIG+BACK}}$) and standard errors ($s_{\bar{X}^{\mathrm{BACK}}}$ and $s_{\bar{X}^{\mathrm{SIG+BACK}}}$). The mean signal is then estimated as

$$\bar{X}^{\mathrm{SIG}} = \bar{X}^{\mathrm{SIG+BACK}} - \bar{X}^{\mathrm{BACK}} \tag{S4}$$

with the standard error estimated via uncertainty propagation as

$$s_{\bar{X}^{\mathrm{SIG}}} \leq \sqrt{(s_{\bar{X}^{\mathrm{SIG+BACK}}})^2 + (s_{\bar{X}^{\mathrm{BACK}}})^2}. \tag{S5}$$

The signal-to-background ratio is estimated as:

$$\bar{X}^{\mathrm{SIG/BACK}} = \bar{X}^{\mathrm{SIG}} / \bar{X}^{\mathrm{BACK}} \tag{S6}$$

with standard error estimated via uncertainty propagation as

$$s^{\mathrm{SIG/BACK}} \leq \bar{X}^{\mathrm{SIG/BACK}} \sqrt{\left(\frac{s_{\bar{X}^{\mathrm{SIG}}}}{\bar{X}^{\mathrm{SIG}}}\right)^2 + \left(\frac{s_{\bar{X}^{\mathrm{BACK}}}}{\bar{X}^{\mathrm{BACK}}}\right)^2}. \tag{S7}$$

These upper bounds on estimated standard errors hold under the assumption that the correlation between SIG and BACK is non-negative. Tables S10–S16 display signal, background, and/or signal-to-background values to characterize the performance of in situ HCR v3.0 within whole-mount chicken embryos.

	Experiment type	Quantity	Reagents: Probes	Reagents: Hairpins	Expression region
A	1	SIG+NSD+NSA+AF = SIG+BACK	odd + even	✓	high
	1	NSD+NSA+AF = BACK	odd + even	✓	no/low
B	2	NSA+AF		✓	high
	3	AF			high
C	4	SIG^{odd}+NSD^{odd}+NSA+AF = SIG^{odd}+$BACK^{odd}$	odd	✓	high
	4	NSD^{odd}+NSA+AF = $BACK^{odd}$	odd	✓	no/low
	5	SIG^{even}+NSD^{even}+NSA+AF = SIG^{even}+$BACK^{even}$	even	✓	high
	5	NSD^{even}+NSA+AF = $BACK^{even}$	even	✓	no/low

Table S5. Experiment types for qHCR imaging using in situ HCR v3.0. (A) Characterize signal, background, and signal-to-background. (B) Characterize components of background (AF, NSA, NSD). (C) Characterize split-initiator HCR suppression.

S1.4.3 Measurement of background components

Calculation of the signal-to-background ratio (Section S1.4.2) requires only a Type 1 experiment (using the terminology of Table S5A), yielding the values $\bar{X}^{\mathrm{SIG+BACK}}$ and $\bar{X}^{\mathrm{BACK}}$ that are needed to calculate SIG/BACK. If desired, additional control experiments that omit certain reagents can be used to characterize the individual components of background (AF, NSA, NSD). A Type 2 experiment (no probes, hairpins only) yields $\bar{X}^{\mathrm{NSA+AF}}$ and a Type 3 experiment (no probes, no hairpins) yields $\bar{X}^{\mathrm{AF}}$.* The background components can then be estimated via calculations analogous to (S4) and (S5). The estimated means are:

$$\bar{X}^{\mathrm{NSD}} = \bar{X}^{\mathrm{BACK}} - \bar{X}^{\mathrm{NSA+AF}} \tag{S8}$$

$$\bar{X}^{\mathrm{NSA}} = \bar{X}^{\mathrm{NSA+AF}} - \bar{X}^{\mathrm{AF}} \tag{S9}$$

with estimated standard errors:

$$s_{\bar{X}^{\mathrm{NSD}}} \leq \sqrt{(s_{\bar{X}^{\mathrm{BACK}}})^2 + (s_{\bar{X}^{\mathrm{NSA+AF}}})^2} \tag{S10}$$

$$s_{\bar{X}^{\mathrm{NSA}}} \leq \sqrt{(s_{\bar{X}^{\mathrm{NSA+AF}}})^2 + (s_{\bar{X}^{\mathrm{AF}}})^2}. \tag{S11}$$

These upper bounds on estimated standard errors hold under the assumption that the correlations are non-negative for the different components of background. For a given quantity, if the estimated mean is less than the estimated standard error, we report the standard error as an upper bound, and use this bound for uncertainty propagation.

Table S14B provides estimates for AF, NSA, and NSD when imaging *EphA4* in whole-mount chicken embryos using in situ HCR v3.0, all of which are small compared to SIG. Kinetically trapped HCR hairpins automatically suppress NSA and the combination of split-initiator probes and HCR hairpins automatically suppress NSD. Furthermore, HCR generates amplified SIG. All of these factors contribute to achieving a high signal-to-background ratio. If a Type 1 experiment demonstrates SIG $\gg$ BACK, as is typically the case using in situ HCR v3.0, then there is little motivation to perform Type 2 and Type 3 experiments to characterize the individual background components (AF, NSA, NSD) as these are all bounded above by BACK.

S1.4.4 Measurement of split-initiator HCR suppression

To characterize performance of in situ HCR v3.0 in suppressing triggering of HCR by individual split-initiator probes, we augment experiments of Type 1 with additional control experiments that omit certain reagents. First, let us define the NSD and SIG observed using odd probes only or even probes only:

*If a microscope generates non-negligible fluorescence intensities in the absence of sample, this so-called instrument noise (NOISE) should be taken into consideration when calculating background and signal contributions, leading to four Experiment Types (1. SIG+BACK+NOISE, 1. BACK+NOISE, 2. NSA+AF+NOISE, 3. AF+NOISE, 4. NOISE; cf. Table S5AB).

- odd non-specific detection (NSD$^{\text{odd}}$): odd probes that bind non-specifically in the sample and subsequently trigger HCR amplification.
- even non-specific detection (NSD$^{\text{even}}$): even probes that bind non-specifically in the sample and subsequently trigger HCR amplification.
- odd signal (SIG$^{\text{odd}}$): odd probes that hybridize specifically to the target mRNA and subsequently trigger HCR amplification.
- even signal (SIG$^{\text{even}}$): even probes that hybridize specifically to the target mRNA in the sample and subsequently trigger HCR amplification.

A Type 4 experiment (odd probes only, with hairpins) yields $\bar{X}^{\text{BACK}^{\text{odd}}+\text{SIG}^{\text{odd}}}$ and $\bar{X}^{\text{BACK}^{\text{odd}}}$ and a Type 5 experiment (even probes only, with hairpins) yields $\bar{X}^{\text{BACK}^{\text{even}}+\text{SIG}^{\text{even}}}$ and $\bar{X}^{\text{BACK}^{\text{even}}}$. These quantities in turn can be used to calculate SIG$^{\text{odd}}$ and SIG$^{\text{even}}$ via calculations analogous to (S4) and (S5). The estimated means are:

$$\bar{X}^{\text{SIG}^{\text{odd}}} = \bar{X}^{\text{SIG}^{\text{odd}}+\text{BACK}^{\text{odd}}} - \bar{X}^{\text{BACK}^{\text{odd}}} \tag{S12}$$

$$\bar{X}^{\text{SIG}^{\text{even}}} = \bar{X}^{\text{SIG}^{\text{even}}+\text{BACK}^{\text{even}}} - \bar{X}^{\text{BACK}^{\text{even}}} \tag{S13}$$

with estimated standard errors:

$$s_{\bar{X}^{\text{SIG}^{\text{odd}}}} \leq \sqrt{(s_{\bar{X}^{\text{SIG}^{\text{odd}}+\text{BACK}^{\text{odd}}}})^2 + (s_{\bar{X}^{\text{BACK}^{\text{odd}}}})^2} \tag{S14}$$

$$s_{\bar{X}^{\text{SIG}^{\text{even}}}} \leq \sqrt{(s_{\bar{X}^{\text{SIG}^{\text{even}}+\text{BACK}^{\text{even}}}})^2 + (s_{\bar{X}^{\text{BACK}^{\text{even}}}})^2} \tag{S15}$$

These upper bounds on estimated standard errors hold under the assumption that the correlations are non-negative for SIG$^{\text{odd}}$ and BACK$^{\text{odd}}$ and for SIG$^{\text{even}}$ and BACK$^{\text{even}}$. For a given quantity, if the estimated mean is less than the estimated standard error, we report the standard error as an upper bound, and use this bound for uncertainty propagation.

Split-initiator HCR suppression can then be characterized by calculating SIG/SIG$^{\text{odd}}$ and SIG/SIG$^{\text{even}}$, with higher values corresponding to more effective suppression. This in situ characterization is akin to the gel studies (Figures 2, S3, and S4) that compare test tube triggering of HCR by odd/even probe pairs colocalized by the target (lane 3) or by either probe alone (lanes 4 or 5). For the in situ data, signal-to-signal ratios are obtained via calculations analogous to (S6) and (S7). The estimated means are:

$$\bar{X}^{\text{SIG/SIG}^{\text{odd}}} = \bar{X}^{\text{SIG}} / \bar{X}^{\text{SIG}^{\text{odd}}} \tag{S16}$$

$$\bar{X}^{\text{SIG/SIG}^{\text{even}}} = \bar{X}^{\text{SIG}} / \bar{X}^{\text{SIG}^{\text{even}}} \tag{S17}$$

with estimated standard errors:

$$s^{\text{SIG/SIG}^{\text{odd}}} \leq \bar{X}^{\text{SIG/SIG}^{\text{odd}}} \sqrt{\left(\frac{s_{\bar{X}^{\text{SIG}}}}{\bar{X}^{\text{SIG}}}\right)^2 + \left(\frac{s_{\bar{X}^{\text{SIG}^{\text{odd}}}}}{\bar{X}^{\text{SIG}^{\text{odd}}}}\right)^2} \tag{S18}$$

$$s^{\text{SIG/SIG}^{\text{even}}} \leq \bar{X}^{\text{SIG/SIG}^{\text{even}}} \sqrt{\left(\frac{s_{\bar{X}^{\text{SIG}}}}{\bar{X}^{\text{SIG}}}\right)^2 + \left(\frac{s_{\bar{X}^{\text{SIG}^{\text{even}}}}}{\bar{X}^{\text{SIG}^{\text{even}}}}\right)^2}. \tag{S19}$$

These upper bounds on estimated standard errors hold under the assumption that the correlations are non-negative for SIG and SIG$^{\text{odd}}$ and for SIG and SIG$^{\text{even}}$.

Table S14C displays the signal-to-signal ratios SIG/SIG$^{\text{odd}}$ and SIG/SIG$^{\text{even}}$ when imaging *EphA4* in whole-mount chicken embryos using in situ HCR v3.0. Split-initiator probes colocalized by the target are more than an order of magnitude more effective at triggering HCR than odd or even probes alone. Interestingly, with this assay, we are quantifying the automatic *background* suppression capabilities of the split-initiator probes by measuring automatic *signal* suppression, taking advantage of the fact that the target molecules in the embryo will colocalize odd/even probe pairs for a Type 1 experiment and will localize odd or even probes to the same expression region for a Type 4 or 5 experiment.

S1.4.5 Normalized voxel intensities for qHCR imaging: analog mRNA relative quantitation with subcellular resolution in an anatomical context

For quantitative mRNA imaging using in situ HCR, precision increases with voxel size as long as the imaging voxels remain smaller than the features in the expression pattern (see Section S2.2 of Trivedi *et al.* (2018)). To increase precision, we calculate raw voxel intensities by averaging neighboring pixel intensities while still maintaining a subcellcular voxel size. To facilitate relative quantitation between voxels, we estimate the normalized HCR signal of voxel j in replicate n as:

$$x_{n,j} \equiv \frac{X_{n,j}^{\mathrm{SIG+BACK}} - X^{\mathrm{BOT}}}{X^{\mathrm{TOP}} - X^{\mathrm{BOT}}}, \tag{S20}$$

which translates and rescales the data so that the voxel intensities in each channel fall in the interval [0,1]. Here,

$$X^{\mathrm{BOT}} \equiv \bar{X}^{\mathrm{BACK}} \tag{S21}$$

is the mean background across replicates (see Section S1.4.2) and

$$X^{\mathrm{TOP}} \equiv \max_{n,j} X_{n,j}^{\mathrm{SIG+BACK}} \tag{S22}$$

is the maximum total fluorescence for a voxel across replicates.

Pairwise expression scatter plots that each display normalized voxel intensities for two channels (e.g., Figures 4 and 5 of Trivedi *et al.* (2018)) provide a powerful quantitative framework for performing multidimensional read-out/read-in analyses (Figure 6 of Trivedi *et al.* (2018)). Read-out from anatomical space to expression space enables discovery of expression clusters of voxels with quantitatively related expression levels and ratios (amplitudes and slopes in the expression scatter plots), while read-in from expression space to anatomical space enables discovery of the corresponding anatomical locations of these expression clusters within the embryo. The simple and practical normalization approach of (S20)–(S22) translates and rescales all voxels identically within a given channel (enabling comparison of amplitudes and slopes in scatter plots between replicates), and does not attempt to remove scatter in the normalized signal estimate that is caused by scatter in the background.

To validate relative mRNA quantitation with subcellular resolution (2×2×2.7 μm voxels) in whole-mount chicken embryos, Figures 5C, S18C, and S19C display highly correlated normalized voxel intensities for 2-channel redundant detection of *Dmbx1* and *EphA4*. In this setting, accuracy corresponds to linearity with zero intercept, and precision corresponds to scatter around the line (Trivedi *et al.*, 2018).

S1.4.6 Dot detection and colocalization for dHCR imaging: digital mRNA absolute quantitation in an anatomical context

To validate the performance of in situ HCR (v3.0) for single-molecule imaging, we perform a 2-channel redundant detection experiment in which a target mRNA is detected using two independent probe sets and HCR amplifiers. Let N_1 denote the number of dots detected in channel 1, N_2 the number of dots detected in channel 2, and N_{12} the number of colocalized dots appearing in both channels. We define the colocalization fraction for each channel:

$$C_1 = N_{12}/N_1, \tag{S23}$$

$$C_2 = N_{12}/N_2. \tag{S24}$$

As the false-positive and false-negative rates for single-molecule detection go to zero, C_1 and C_2 will both approach 1 from below, providing a quantitative basis for evaluating performance. Colocalization results using in situ HCR v3.0 with split-initiator probes (23-25 probe pairs per channel) in cultured human cells (Table S25) and whole-mount chicken embryos (Table S26) are ≈84%, compared with ≈50% using in situ HCR v2.0 (39 standard probe pairs per channel) in a previous study in whole-mount zebrafish embryos (Table S27)(Shah *et al.*, 2016).

Single molecules were identified in each channel using the following dot detection algorithm applied to a three-dimensional confocal image stack:

- **Step 1: Blur noise.** To remove noise smaller than the dots of interest, the image was convolved with an isotropic Gaussian blur (standard deviation σ_{blur}).
- **Step 2: Local background subtraction.** To eliminate variations in pixel intensity arising from background variations that occur on a length scale larger than the dots of interest, local background subtraction was performed by subtracting the mean pixel intensity of a cube (edge length d_{back}) from the intensity of the pixel at the center of the cube.
- **Step 3: Global threshold on pixel intensity.** To eliminate dim features, the resulting pixel intensities were subjected to a global threshold (t_{pixel}), and the range [t_{pixel},1] was renormalized to a [0,1] scale.
- **Step 4: Watershed dot detection.** To identify single mRNA molecules as dots within the image, regional image maxima were segmented using the minimum saliency watershed method (Couprie & Bertrand, 1997; Yoo *et al.*, 2002). In this method, two maxima are labeled as the same dot if the minimum boundary height between the maxima is less than a given threshold ($t_{\mathrm{watershed}}$). Dot coordinates were estimated as the intensity-weighted centroid of each watershed basin, and dot intensities were estimated as the integrated pixel intensity within each basin.
- **Step 5: Global threshold on dot intensity.** To eliminate dim dots, the resulting dot intensities were normalized on a [0,1] scale and a global threshold (t_{dot}) was applied. The resulting number of dots N_i was recorded for channel i.

After identifying the dots in each channel of a 2-channel redundant detection image, a dot i in Channel 1 and a dot j in Channel 2 were considered colocalized if all of the following four statements were true:

- **Test 1:** The xy centroids differed by less than a lateral distance threshold ($r_{xy} = 0.22$ μm).
- **Test 2:** Dot i is the closest dot in Channel 1 to dot j in Channel 2.
- **Test 3:** Dot j is the closest dot in Channel 2 to dot i in Channel 1.
- **Test 4:** The z centroids differed by less than the axial distance threshold ($r_z = 0.42$ μm).

Note that due to the lower axial resolution, dot colocalization was tested separately for xy and z. The same distance thresholds were used across all sample types and replicates.[†]

[†]For chicken embryo replicate 3, Channels 1 and 2 were manually aligned by applying a constant offset of $+0.35\mu$m to the z-coordinates of Channel 1 after the detected dots showed a clear bias in z coordinates between the two channels. No other images were manually aligned.

		HEK cells		Chicken embryos		Zebrafish embryos	
lateral resolution d_{xy} (μm)		0.0624		0.099		0.2167	
axial resolution d_z (μm)		0.42		0.42		0.3369	
Step 1: Gaussian blur radius σ_{blur} (μm)		0.2		0.2		0.1	
Step 2: Mean subtraction cube length d_{back} (μm)		2.5		3.0		5.5	
	Replicate	Ch1	Ch2	Ch1	Ch2	Ch1	Ch2
Step 3: Global pixel threshold (t_{pixel})	1	0.003	0.012	0.04	0.02	0.02	0.02
	2	0.005	0.012	0.04	0.02	0.01	0.003
	3	0.004	0.01	0.04	0.02	0.01	0.005
Step 4: Watershed saliency minimum ($t_{\mathrm{watershed}}$)	1	0.15	0.15	0.15	0.15	0.15	0.15
	2	0.15	0.15	0.15	0.15	0.30	0.20
	3	0.15	0.15	0.15	0.15	0.35	0.30
Step 5: Global dot intensity threshold (t_{dot})	1	0.01	0.005	0.00	0.01	0.01	0.01
	2	0.02	0.02	0.006	0.013	0.025	0.025
	3	0.03	0.012	0.01	0.025	0.04	0.04

Table S6. Parameters used for dot detection in dHCR images.

S1.5 Flow cytometry data analysis

Data analysis for flow cytometry experiments on cultured cells closely follows the image analysis of Section S1.4 as detailed below.

S1.5.1 Raw cell intensities

The components of background (AF, NSA, NSD) and signal (SIG) are defined as before (Section S1.4.1), where n is treated as an index over cells, and $i = 1$ for each cell since the flow cytometer returns one value per cell.

S1.5.2 Measurement of signal, background, signal-to-background, background components, and split-initiator HCR suppression for transgenic targets

For a transgenic target mRNA, signal and background are characterized based on flow cytometry experiments of Types 1a and 1b (Table S7A) with SIG+BACK measured in transgenic cells containing the target and BACK measured in wildtype (WT) cells lacking the target. This approach parallels that for characterizing signal and background in images (Section S1.4.2) with transgenic cells taking the place of a region of high expression and WT cells taking the place of a region of no/low expression. If desired, additional control experiments of Types 2 and 3 (Table S7B) can be performed to characterize the components of background (AF, NSA, NSD) using the calculations of Section S1.4.3. Likewise, additional control experiments of Types 4a, 4b, 5a, and 5b (Table S7C) can be performed to characterize split-initiator HCR suppression using the calculations of Section S1.4.4. For transgenic target mRNAs in human and bacterial cells, Figures S20 and S22 display distributions of cell intensities characterizing signal and background (panel A) and split-initiator HCR suppression (panel B). Tables S17 and S19 display corresponding values for SIG, BACK, and SIG/BACK (panel A), background components AF, NSA, and NSD (panel B), and SIG/SIG$^{\text{odd}}$ and SIG/SIG$^{\text{even}}$ (panel C).

			Reagents		
	Experiment type	Quantity	Probes	Hairpins	Cell type
A	1a	SIG+NSD+NSA+AF = SIG+BACK	odd + even	✓	transgenic
	1b	NSD+NSA+AF = BACK	odd + even	✓	WT
B	2	NSA+AF		✓	transgenic
	3	AF			transgenic
C	4a	SIG$^{\text{odd}}$+NSD$^{\text{odd}}$+NSA+AF = SIG$^{\text{odd}}$+BACK$^{\text{odd}}$	odd	✓	transgenic
	4b	NSD$^{\text{odd}}$+NSA+AF = BACK$^{\text{odd}}$	odd	✓	WT
	5a	SIG$^{\text{even}}$+NSD$^{\text{even}}$+NSA+AF = SIG$^{\text{even}}$+BACK$^{\text{even}}$	even	✓	transgenic
	5b	NSD$^{\text{even}}$+NSA+AF = BACK$^{\text{even}}$	even	✓	WT

Table S7. Experiment types for flow cytometry using in situ HCR v3.0 with a transgenic target mRNA. (A) Characterize signal, background, and signal-to-background. (B) Characterize components of background (AF, NSA, NSD). (C) Characterize split-initiator HCR suppression.

S1.5.3 Measurement of signal, background, signal-to-background, background components, and split-initiator HCR suppression for endogenous targets

For an endogenous target mRNA, signal and background are characterized based on flow cytometry experiments of Types 1a and 1b (Table S8A) with SIG+BACK measured using a probe set (odd + even) that address the target in WT cells and BACK measured using a probe set (Tg(odd) and Tg(even)) that addresses a different transgenic target absent from WT cells. Use of a previously validated transgenic probe set to measure background in WT cells ensures that a low measured fluorescence value does not simply indicate a dysfunctional probe set, but indeed represents low background generated by a probe set that is known to be functional if the target is present in the sample. If desired,

	Experiment type	Quantity	Reagents: Probes	Reagents: Hairpins	Cell type
A	1a	SIG+NSD+NSA+AF = SIG+BACK	odd + even	✓	WT
	1b	NSD+NSA+AF = BACK	Tg(odd) + Tg(even)	✓	WT
B	2	NSA+AF		✓	WT
	3	AF			WT
C	4a	SIG^{odd}+NSD^{odd}+NSA+AF = SIG^{odd}+$BACK^{odd}$	odd	✓	WT
	4b	NSD^{odd}+NSA+AF = $BACK^{odd}$	Tg(odd)	✓	WT
	5a	SIG^{even}+NSD^{even}+NSA+AF = SIG^{even}+$BACK^{even}$	even	✓	WT
	5b	NSD^{even}+NSA+AF = $BACK^{even}$	Tg(even)	✓	WT

Table S8. Experiment types for flow cytometry using in situ HCR v3.0 with an endogenous target mRNA. (A) Characterize signal, background, and signal-to-background. (B) Characterize components of background (AF, NSA, NSD). (C) Characterize split-initiator HCR suppression. Here, Tg(odd) and Tg(even) denote odd and even probes from a probe set targeting a transgenic mRNA that is absent from WT cells.

additional control experiments of Types 2 and 3 (Table S8B) can be performed to characterize the components of background (AF, NSA, NSD) using the calculations of Section S1.4.3. Likewise, additional control experiments of Types 4a, 4b, 5a, and 5b (Table S8C) can be performed to characterize split-initiator HCR suppression using the calculations of Section S1.4.4. For endogenous target mRNA *GAPDH*, Figure S21 displays distributions of cell intensities characterizing signal and background (panel A) and split-initiator HCR suppression (panel B). Table S18 displays corresponding values for SIG, BACK, and SIG/BACK (panel A), background components AF, NSA, and NSD (panel B), and SIG/SIG^{odd} and SIG/SIG^{even} (panel C).

S1.5.4 Normalized single-cell intensities for qHCR flow cytometry: analog mRNA relative quantitation for high-throughput expression profiling of human and bacterial cells

For relative mRNA quantitation between cells, the single-cell intensities within a channel are normalized using equation (S20) with BOT (mean BACK intensity across cells) and TOP (maximum SIG+BACK intensity for a single cell) defined by equations (S21) and (S22). Redundant detection experiments validating mRNA single-cell relative quantitation are displayed for endogenous targets *GAPDH* (Figure S24 and Table S20) and *PGK1* (Figure S25 and Table S21) in HEK cells, and for endogenous target *fusA* in *E. coli* (Figure S26 and Table S22).

S2 Protocols for in situ HCR v3.0

S2.1 Protocols for whole-mount chicken embryos

S2.1.1 Preparation of fixed whole-mount chicken embryos

1. Collect chicken embryos on 3M filter paper and place in a petri dish containing Ringer's solution.
2. Transfer embryos into a new petri dish with fresh Ringer's solution.
   NOTE: *This is to rinse away egg yolk before fixation.*
3. Transfer into a petri dish containing 4% paraformaldehyde (PFA).
   CAUTION: *Use PFA with extreme care as it is a hazardous material.*
   NOTE: *Use fresh PFA to avoid increased autofluorescence.*
4. Fix the samples at room temperature for 1 h.
5. Transfer embryos into a petri dish containing PBST.
6. Dissect the embryos off the filter paper. Remove the fixed vitelline membrane (opaque) and cut the squares around area pellucida, without leaving excess of extra-embryonic tissue.
7. Transfer embryos into a 2 mL Eppendorf tube containing PBST.
8. Nutate for 5 mins with the tube positioned horizontally in a small ice bucket.
9. Wash embryos two additional times with 2 mL of PBST, each time nutating for 5 min on ice.
10. Dehydrate embryos with 2 × 5 min washes of 2 mL methanol (MeOH) on ice.
11. Store embryos at -20 °C overnight before use.
    NOTE: *Embryos can be stored for six months at -20 °C.*
12. Transfer the required number of embryos for an experiment to a 2 mL Eppendorf tube.
    NOTE: *Do not place more than 4 embryos in each 2 mL Eppendorf tube.*
13. Rehydrate with a series of graded 2 mL MeOH/PBST washes for 5 min each on ice:
    (a) 75% MeOH / 25% PBST
    (b) 50% MeOH / 50% PBST
    (c) 25% MeOH / 75% PBST
    (d) 100% PBST
    (e) 100% PBST.
14. Treat embryos with 2 mL of 10 μg/mL proteinase K solution for 2 min (stage HH 8) or 2.5 min (stages HH 10–11) at room temperature.
    NOTE: *Proteinase K concentration and treatment time should be reoptimized for each batch of proteinase K, or for samples at a different developmental stage.*
15. Postfix with 2 mL of 4% PFA for 20 min at room temperature.
16. Wash embryos 2 × 5 min with 2 mL of PBST on ice.
17. Wash embryos with 2 mL of 50% PBST / 50% 5× SSCT for 5 min on ice.
18. Wash embryos with 2 mL of 5× SSCT for 5 min on ice.

S2.1.2 Buffer recipes for sample preparation

Ringer's solution	For 2 L of solution
123 mM NaCl	14.4 g of NaCl
1.53 mM $CaCl_2$	340 mg of $CaCl_2$
4.96 mM KCl_2	740 mg of KCl
0.81 mM Na_2HPO_4	230 mg of Na_2HPO_4
0.15 mM KH_2PO_4	40 mg of KH_2PO_4
	Bring volume up to 1.5 L with ultrapure H_2O
	Adjust pH to 7.4 and fill up to 2 L with ultrapure H_2O
	Filter sterilize with 0.22 μm bottle top filter

4% Paraformaldehyde (PFA)	For 25 mL of solution
4% PFA	1 g of PFA powder
1× PBS	25 mL of 1× PBS
	Heat solution at 50–60 °C to dissolve powder

PBST	For 50 mL of solution
1× PBS	5 mL of 10× PBS
0.1% Tween 20	500 μL of 10% Tween 20
	Fill up to 50 mL with ultrapure H_2O

Proteinase K solution	For 2 mL of solution
10 μg/mL proteinase K	1 μL of 20 mg/mL proteinase K
	Fill up to 2 mL with PBST

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

S2.1.3 Multiplexed in situ HCR v3.0 using split-initiator probes

Detection stage

1. For each sample, transfer 1-4 embryos to a 2 mL Eppendorf tube.
   NOTE: *Do not place more than 4 embryos in each 2 mL Eppendorf tube.*

2. Incubate embryos in 1 mL of 30% probe hybridization buffer on ice for 5 min.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
   NOTE: *Pre-heat probe hybridization buffer to 37 °C before use.*

3. Remove the buffer and pre-hybridize with 1 mL of 30% probe hybridization buffer for 30 min at 37 °C.

4. Prepare probe solution by adding 4 pmol of each probe mixture (odd & even: 2 μL of 2 μM stock per probe mixture) to 1 mL of 30% probe hybridization buffer at 37 °C.
   NOTE: *For Figures 7B and S30 (dHCR), 10 pmol of each probe was used to improve probe hybridization efficiency.*

5. Remove the pre-hybridization solution and add the probe solution.

6. Incubate embryos overnight (12–16 h) at 37 °C.

7. Remove excess probes by washing embryos 4 × 15 min with 1 mL of 30% probe wash buffer at 37 °C:
   CAUTION: *probe wash buffer contains formamide, a hazardous material.*
   NOTE: *Pre-heat probe wash buffer to 37 °C before use.*

8. Wash samples 2 × 5 min with 5× SSCT at room temperature.

Amplification stage

1. Pre-amplify embryos with 500 mL of amplification buffer for 5 min at room temperature.
   NOTE: *Equilibrate amplification buffer to room temperature before use.*

2. Prepare 30 pmol of each fluorescently labeled hairpin 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).

3. Prepare hairpin solution by adding all snap-cooled hairpins to 500 μL of amplification buffer at room temperature.

4. Remove the pre-amplification solution and add the hairpin solution.

5. Incubate the embryos overnight (12–16 h) in the dark at room temperature.
   NOTE: *For Figures 7B and S30 (dHCR), a 90 min amplification time was used to ensure single-molecule dots are diffraction-limited.*

6. Remove excess hairpins by washing with 1 mL of 5× SSCT at room temperature:

   (a) 2 × 5 min
   (b) 2 × 30 min
   (c) 1 × 5 min

S2.1.4 Buffer recipes for in situ HCR v3.0

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.

30% probe hybridization 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 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% dextran sulfate	8 mL of 50% 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 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	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% dextran sulfate	8 mL of 50% 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 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 dextran sulfate powder
	Fill up to 40 mL with ultrapure H_2O

S2.1.5 Sample mounting for microscopy

1. Make a chamber for mounting each embryo by aligning two stacks of double-sided tape (2 pieces per stack) 1 cm apart on a 25 mm $\times$ 75 mm glass slide.
2. Place an embryo between the tape stacks on the slide and remove as much solution as possible.
3. Align the embryo for dorsal imaging and carefully touch the slide with a kimwipe to further dry the area around the embryo.
4. Add two drops of SlowFade Gold antifade mountant on top of the embryo.
5. Place a 22 mm $\times$ 30 mm No. 1 coverslip on top of the stacks to close the chamber.
   NOTE: *See Section S1.2 for details of confocal microscopes used to image whole-mount chicken embryos.*

S2.1.6 Reagents and supplies

Paraformaldehyde (PFA) (Sigma Cat. # P6148)
Methanol (Mallinckrodt Chemicals Cat. # 3016-16)
Proteinase K, molecular biology grade (NEB Cat. # P8107S)
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. $>$ 500,000 (Sigma Cat. # D6001)
25 mm × 75 mm glass slide (VWR Cat. # 48300-025)
22 mm × 30 mm No. 1 coverslip (VWR Cat. # 48393-026)
SlowFade Gold antifade mountant (Life Technologies Cat. # S36937)

S2.2 Protocols for mammalian cells on a chambered slide

S2.2.1 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.*
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.
11. Permeabilize cells overnight at -20 °C.
12. Cells can be stored at -20 °C or 4 °C until use.

S2.2.2 Buffer recipes for sample preparation

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

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

S2.2.3 Multiplexed in situ HCR v3.0 using split-initiator probes

Detection stage

1. Aspirate EtOH and air dry samples at room temperature.
   NOTE: *Drying of sample is optional.*
2. Wash samples two times with 300 μL of 2× SSC.
3. Pre-hybridize samples in 300 μL of 30% probe hybridization buffer for 30 min at 37 °C.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
   NOTE: *Pre-heat probe hybridization buffer to 37 °C before use.*
4. Prepare probe solution by adding 1.2 pmol of each probe mixture (odd & even: 0.6 μL of 2 μM stock per probe mixture) to 300 μL of 30% probe hybridization buffer at 37 °C.
   NOTE: *For Figures 7A and S29 (dHCR), 3 pmol of each probe was used to improve probe hybridization efficiency.*
5. Remove the pre-hybridization solution and add the probe solution.
6. Incubate samples overnight (12–16 h) at 37 °C.
7. Remove excess probes by washing 4 × 5 min with 300 μL of 30% probe wash buffer at 37 °C.
   CAUTION: *probe wash buffer contains formamide, a hazardous material.*
   NOTE: *Pre-heat probe wash buffer to 37 °C before use.*
8. Wash samples 2 × 5 min with 5× SSCT at room temperature.

Amplification stage

1. Pre-amplify samples in 300 μL of amplification buffer for 30 min at room temperature.
   NOTE: *Equilibrate amplification buffer to room temperature before use.*
2. Prepare 18 pmol of each fluorescently labeled hairpin by snap cooling 6 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).
3. Prepare hairpin solution by adding all snap-cooled hairpins to 300 μL of amplification buffer at room temperature.
4. Remove the pre-amplification solution and add the hairpin solution.
5. Incubate samples overnight (12–16 h) in the dark at room temperature.
   NOTE: *For Figures 7A and S29 (dHCR), a 45 min amplification time was used to ensure single-molecule dots are diffraction-limited.*
6. Remove excess hairpins by washing 5 × 5 min with 300 μL of 5× SSCT at room temperature.
7. Aspirate 5× SSCT and add ≈100 μL of SlowFade Gold antifade mountant with DAPI.
8. Samples can be stored at 4 °C protected from light prior to imaging.

S2.2.4 Buffer recipes for in situ HCR v3.0

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.

30% probe hybridization 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 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% dextran sulfate	8 mL of 50% 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 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	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% dextran sulfate	8 mL of 50% 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 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 dextran sulfate powder
	Fill up to 40 mL with ultrapure H_2O

S2.2.5 Reagents and supplies

Molecular biology grade H_2O (Corning Cat. # 46-000-CV)
16% Formaldehyde (w/v), Methanol-free (Life Technologies Cat. # 28906)
DPBS, no calcium, no magnesium (Life Technologies Cat. # 14190144)
10× PBS (Ambion Cat. # AM9624)
Formamide (Deionized) (Ambion Cat. # AM9342)
20× sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)
Heparin (Sigma Cat. # H3393)
10% Tween 20 (BioRad Cat. # 161-0781)
50× Denhardt's solution (Life Technologies Cat. # 750018)
Dextran sulfate, mol. wt. $>$ 500,000 (Sigma Cat. # D6001)
ibidi μ-slide ibitreat (ibidi Cat. # 80826)
SlowFade Gold antifade mountant with DAPI (Life Technologies Cat. # S36938)

S2.3 Protocols for mammalian cells in suspension

S2.3.1 Preparation of fixed mammalian cells in suspension

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 for 5 min at 37 °C.
3. Quench trypsin by adding 3 mL of growth media.
4. Transfer cells to a conical tube and centrifuge for 5 min at 180 × g.
5. Aspirate supernatant and re-suspend cells in 4% formaldehyde to reach $\approx 10^6$ cells/mL.
   CAUTION: *Use formaldehyde with extreme care as it is a hazardous material.*
6. Fix cells for 1 hr at room temperature.
7. Centrifuge for 5 min at 180 × g and remove supernatant.
8. Wash cells 4 times with PBST (use the same volume as formaldehyde solution).
   NOTE: *Centrifuge for 5 min at 180 × g and aspirate supernatant between washes.*
9. Re-suspend cells in ice-cold 70% ethanol (use the same volume as formaldehyde and PBST solutions).
10. Permeabilize cells overnight at 4 °C.
11. Cells can be stored at 4 °C until use.

S2.3.2 Buffer recipes for sample preparation

4% formaldehyde in PBST	For 36 mL of solution
4% formaldehyde	9 mL of 16% formaldehyde
1× PBS, 0.1% Tween 20	3.6 mL of 10× PBST
	180 μL of 10% Tween 20
	Fill up to 36 mL with ultrapure H_2O

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

S2.3.3 Multiplexed in situ HCR v3.0 using split-initiator probes

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 to remove EtOH.
   NOTE: *All centrifugation steps are done at 180 $\times$ g.*
3. Wash cells twice with 500 μL of PBST. Centrifuge for 5 min to remove supernatant.
4. Re-suspend the pellet with 400 μL of 30% LMW probe hybridization buffer and pre-hybridize for 30 min at 37 °C.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
   NOTE: *Pre-heat probe hybridization buffer to 37 °C before use.*
5. In the meantime, prepare probe solution by adding 2 pmol of each probe mixture (odd & even: 1 μL of 2 μM stock per probe mixture) to 100 μL of 30% LMW probe hybridization buffer at 37 °C.
6. Add the probe solution directly to the sample to reach a final probe concentration of 4 nM.
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 μL of 30% probe wash buffer.
   CAUTION: *probe wash buffer contains formamide, a hazardous material.*
   NOTE: *Pre-heat probe wash buffer 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 μL 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 μL of LMW amplification buffer and pre-amplify for 30 min at room temperature.
   NOTE: *Equilibrate amplification buffer to room temperature before use.*
3. Prepare 15 pmol of each fluorescently labeled hairpin by snap cooling 5 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).
4. Prepare hairpin mixture by adding all snap-cooled hairpins to 100 μL of LMW 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.
7. Centrifuge for 5 min and remove the hairpin solution.

8. Re-suspend the cell pellet with 500 μL 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.
    NOTE: *Samples can be stored at 4 °C protected from light before flow cytometry or imaging.*
12. Filter cells before flow cytometry.

S2.3.4 Buffer recipes for in situ HCR v3.0

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.

30% probe hybridization buffer (LMW d.s.)	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 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

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 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 (LMW d.s.)	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

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

1× PBST	For 40 mL of solution
1× PBS	10 mL of 10× PBST (0.5% Tween 20)
0.1% Tween 20	200 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

S2.3.5 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)

S2.4 Protocols for bacteria in suspension

S2.4.1 Preparation of fixed bacteria in suspension

1. Grow *E. coli* from streaked plate or frozen glycerol stocks in 2–3 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 a 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 1 × phosphate-buffered saline (PBS).
   NOTE: *Remove all solutions via pipetting throughout the protocol.*
6. Add 250 μL of 4% formaldehyde to 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 1 × PBS.
9. Add 850 μL of 100% MeOH and store at -20 °C before use.

S2.4.2 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 in PBS

4% formaldehyde
1× PBS

For 4 mL of solution
1 mL of 16% formaldehyde
0.4 mL of 10× PBS
Fill up to 4 mL with ultrapure H_2O

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

S2.4.3 Multiplexed in situ HCR v3.0 using split-initiator probes

Detection stage

1. Transfer 150 μL of fixed 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. Centrifuge for 5 min to remove supernatant.
4. Re-suspend the pellet with 400 μL of 30% LMW probe hybridization buffer and pre-hybridize for 1 hr at 37 °C.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
   NOTE: *Pre-heat probe hybridization buffer to 37 °C before use.*
5. In the meantime, prepare probe solution by adding 2 pmol of each probe mixture (odd & even: 1 μL of 2 μM stock per probe mixture) to 100 μL of 30% LMW probe hybridization buffer at 37 °C.
6. Add the probe solution directly to the sample to reach a final probe concentration of 4 nM.
7. Incubate the sample overnight at 37 °C.
8. Add 1mL of probe wash buffer to the sample.
   CAUTION: *probe wash buffer contains formamide, a hazardous material.*
   NOTE: *Pre-heat probe wash buffer to 37 °C before use.*
9. Centrifuge for 5 min and remove the wash solution.
10. Re-suspend the cell pellet with 500 μL wash solution.
11. Incubate for 5 min at 37 °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 150 μL of LMW amplification buffer and pre-amplify for 30 min at room temperature.
   NOTE: *Equilibrate amplification buffer to room temperature before use.*
2. Prepare 15 pmol of each fluorescently labeled hairpin by snap cooling 5 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).
3. Prepare hairpin mixture by adding all snap-cooled hairpins to 100 μL of LMW 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 solution.
7. Centrifuge for 5 min and remove the hairpin solution.
8. Re-suspend the cell pellet with 500 μL of 5$\times$ SSCT and incubate 5 min at room temperature.
9. Centrifuge for 5 min and remove the wash solution.

10. Repeat steps 8 and 9 for two additional times but with a 10 min incubation.

11. Re-suspend the cell pellet in desired buffer and volume.
NOTE: *Samples can be stored at 4 °C protected from light before flow cytometry.*

12. Filter cells before flow cytometry.

S2.4.4 Buffer recipes for in situ HCR v3.0

Probes, amplifiers, probe hybridization buffer, and probe wash buffer should be stored at -20 °C. Amplification buffer should be stored at 4 °C. Make sure all solutions are well mixed before use.

30% probe hybridization buffer (low MW D. S.)	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 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

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 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 D. S.)	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

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

1× PBST	For 40 mL of solution
1× PBS	10 mL of 10× PBST (0.5% Tween 20)
0.1% Tween 20	200 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

S2.4.5 Reagents and supplies

LB Broth Miller (Novagen Cat. # 71753-5)
16% Formaldehyde (w/v), methanol-free (Life Technologies Cat. # 28908)
10× PBS (Ambion Cat. # AM9624)
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)

S3 Additional studies

S3.1 Validation of split-initiator HCR suppression in vitro and in situ (cf. Figure 2)

Figures S3 and S4 display gel studies measuring split-initiator HCR suppression for amplifiers B1–B5, revealing typical ≈60-fold suppression. Table S9 displays signal-to-signal ratios for the same amplifiers used in situ within whole-mount chicken embryos (imaging) and/or within cultured human or bacterial cells (flow cytometry), revealing typical ≈50-fold suppression.

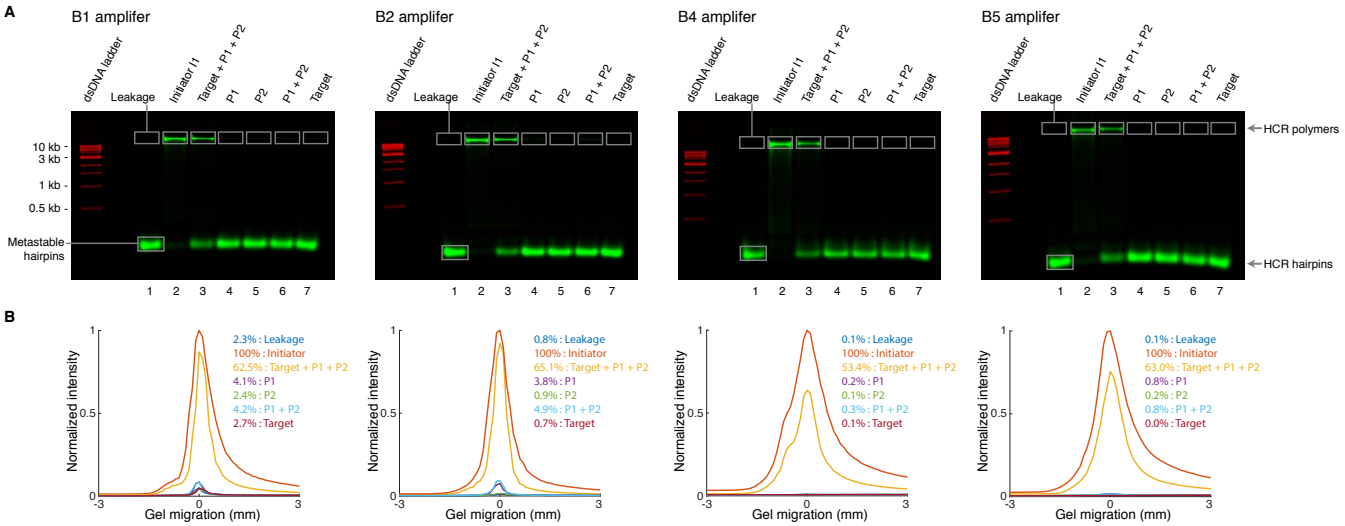

Figure S3. Test tube validation of split-initiator HCR suppression for amplifiers B1, B2, B4, and B5 (cf. Figure 2). (A) Agarose gel electrophoresis. Reaction conditions: hairpins H1 and H2 at 0.5 μM each (Lanes 1-7); DNA oligos I1, P1, P2, and/or Target at 5 nM each (lanes noted on the gel); 5× SSCT buffer; overnight reaction at room temperature. Hairpins H1 and H2 labeled with Alexa 647 fluorophore (green channel). dsDNA 1 kb ladder pre-stained with SYBR Gold (red channel). (B) Quantification of the polymer band in panel (A).

Organism	Target	Samples	Channel	SIG/SIG$^{\text{odd}}$	SIG/SIG$^{\text{even}}$	Table
G. gallus domesticus	*EphA4*	3 embryos	B2-Alexa647	>800	57 ± 5	S14
H. sapiens sapiens	*Tg(d2eGFP)*	55,000 cells	B3-Alexa594	461 ± 9	22.2 ± 0.1	S17
H. sapiens sapiens	*GAPDH*	30,000 cells	B4-Alexa594	55 ± 5	42.9 ± 0.6	S18
H. sapiens sapiens	*GAPDH*	20,000 cells	B5-Alexa488	40.4 ± 0.8	>3000	S20
H. sapiens sapiens	*GAPDH*	20,000 cells	B4-Alexa594	67 ± 2	52 ± 1	S20
H. sapiens sapiens	*PGK1*	54,000 cells	B1-Alexa488	>5000	49.0 ± 0.5	S21
H. sapiens sapiens	*PGK1*	54,000 cells	B2-Alexa594	41 ± 1	13 ± 1	S21
H. sapiens sapiens	*GAPDH*	18,000 cells	B4-Alexa488	93 ± 6	91 ± 14	S23
H. sapiens sapiens	*PGK1*	18,000 cells	B2-Alexa594	21 ± 2	18.8 ± 0.4	S23
E. coli	*Tg(eGFP)*	18,000 cells	B3-Alexa594	>3000	9.9 ± 0.5	S19
E. coli	*fusA*	3,400 cells	B3-Alexa488	40 ± 20	14 ± 3	S22
E. coli	*fusA*	3,400 cells	B2-Alexa594	50 ± 20	6.2 ± 0.6	S22
E. coli	*fusA*	35,000 cells	B3-Alexa488	600 ± 300	17.2 ± 1.3	S24
E. coli	*icd*	35,000 cells	B1-Alexa594	800 ± 300	85 ± 4	S24

Table S9. In situ validation of split-initiator HCR suppression.

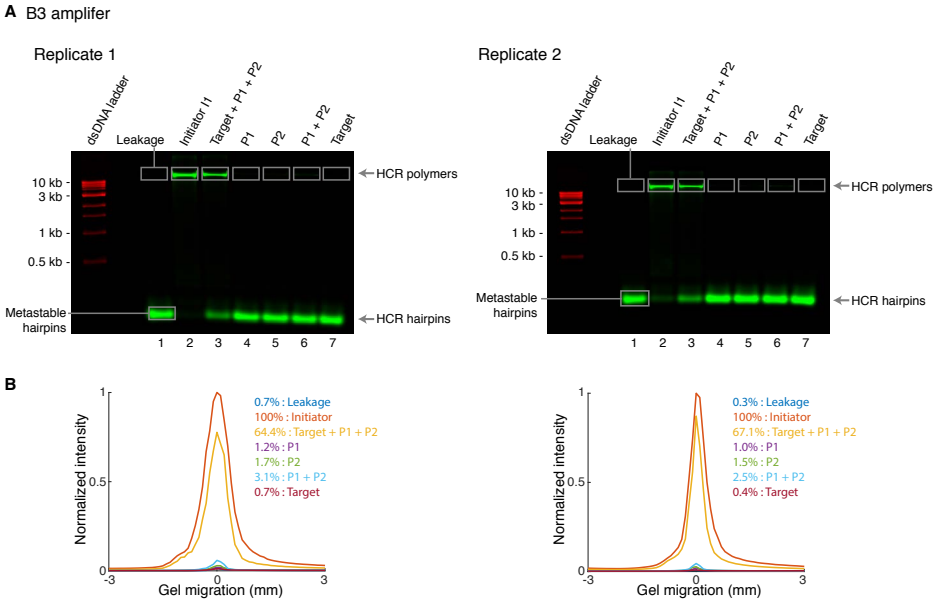

Figure S4. Test tube validation of split-initiator HCR suppression for amplifier B3 (cf. Figure 2). (A) Agarose gel electrophoresis (Replicate 1 is displayed in Figure 2). Reaction conditions: hairpins H1 and H2 at 0.5 μM each (Lanes 1-7); DNA oligos I1, P1, P2, and/or Target at 5 nM each (lanes noted on the gel); 5× SSCT buffer; overnight reaction at room temperature. Hairpins H1 and H2 labeled with Alexa 647 fluorophore (green channel). dsDNA 1 kb ladder pre-stained with SYBR Gold (red channel). (B) Quantification of the polymer band in panel (A).

S3.2 In situ validation of automatic background suppression with split-initiator probes in whole-mount chicken embryos (cf. Figure 3)

The following studies are included:

- **Measurement of background and signal-to-background for unoptimized standard and split-initiator probe sets as a function of probe set size.** Probe sets with 5, 10, or 20 standard probes or split-initiator probe pairs. For standard probes, the probe set size is increased from 5 to 10 to 20 by adding untested probes to a previously validated set of 5 probes Choi *et al.* (2016); each standard probe has a 50-nt target-binding site and carries two HCR initiators (one at each end). Each split-initiator probe pair addresses the nearly identical target subsequence as the corresponding standard probe; each split-initiator probe has a 25-nt target-binding site and carries half an HCR initiator; the two probes within a pair are separated by 2 nt along the target so the overall target binding site for a probe pair is 52 nt. For background comparisons (Figures S5 and S6 and Table S10), the PMT gain is held constant for all experiments to enable comparison of background intensities between experiments. For signal-to-background comparisons (Figures S7 and S8 and Table S11), the PMT gain is adjusted to use the full dynamic range for each probe set.
- **Measurement of background and signal-to-background for standard and split-initiator probes with identical target-binding domains.** For these studies, standard probes are constructed from split-initiator probe pairs as follows. For each split-initiator probe pair in a probe set, the full initiator is shifted onto either the odd probe (full-initiator odd probe + initiator-free even probe) or onto the even probe (initiator-free odd probe + full-initiator even probe). Within each pair, one probe is then a full-initiator standard probe and one probe is a helper probe that contains no initiator. The helper probes are employed to ensure that each standard probe pair (full-initiator probe + helper probe) has the same target-binding capabilities as its analogous split-initiator probe pair. Probe sets with 20 standard probe pairs or split-initiator probe pairs. For background comparisons (Figure S9 and Table S12), the PMT gain is held constant for all experiments to enable comparison of background intensities between experiments. For signal-to-background comparisons (Figure S10 and Table S13), the PMT gain is adjusted to use the full dynamic range for each probe set.
- **Measurement of signal, background, and signal-to-background, background components, and split-initiator HCR suppression.** Measurement of signal, background, and signal-to-background (Figure S11A and Table S14A) using the methods of Section S1.4.2. Measurement of background components (AF, NSA, NSD; Figure S11B and Table S14B) using the methods of Section S1.4.3. Measurement of split-initiator HCR suppression (Figure S11C and Table S14C) using the methods of Section S1.4.4.

S3.2.1 Measurement of background and signal-to-background for unoptimized standard and split-initiator probe sets as a function of probe set size

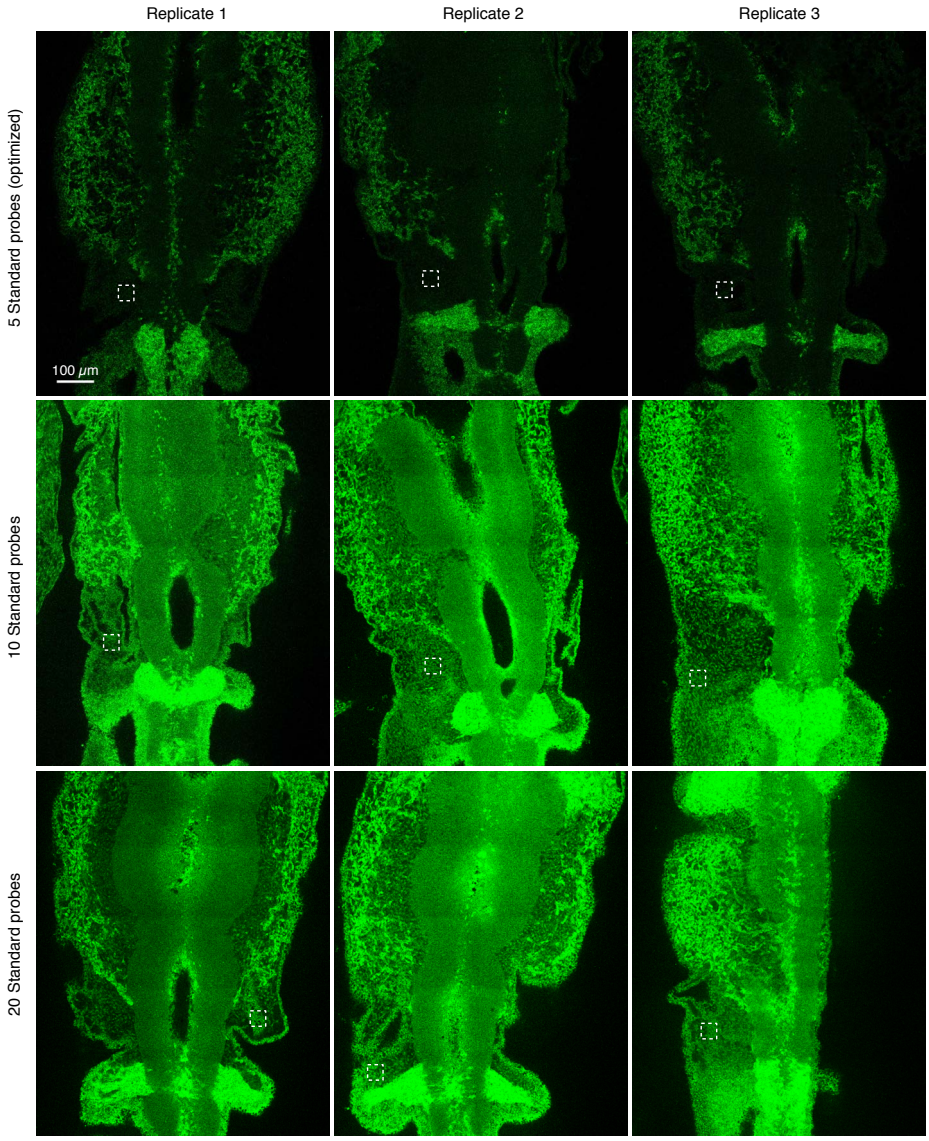

Figure S5. Measurement of background for unoptimized standard probe sets as a function of probe set size (cf. Figure 3A). Confocal images collected with the microscope PMT gain adjusted to avoid saturating pixels using 20 standard probes (row 3). Protocol: in situ HCR v2.0 (Section S8 of Choi *et al.* (2016)). Target mRNA: *Sox10*. Probe set: 5, 10, or 20 standard probes. Amplifier: B3-Alexa647. Whole-mount chicken embryos fixed stage HH 11.

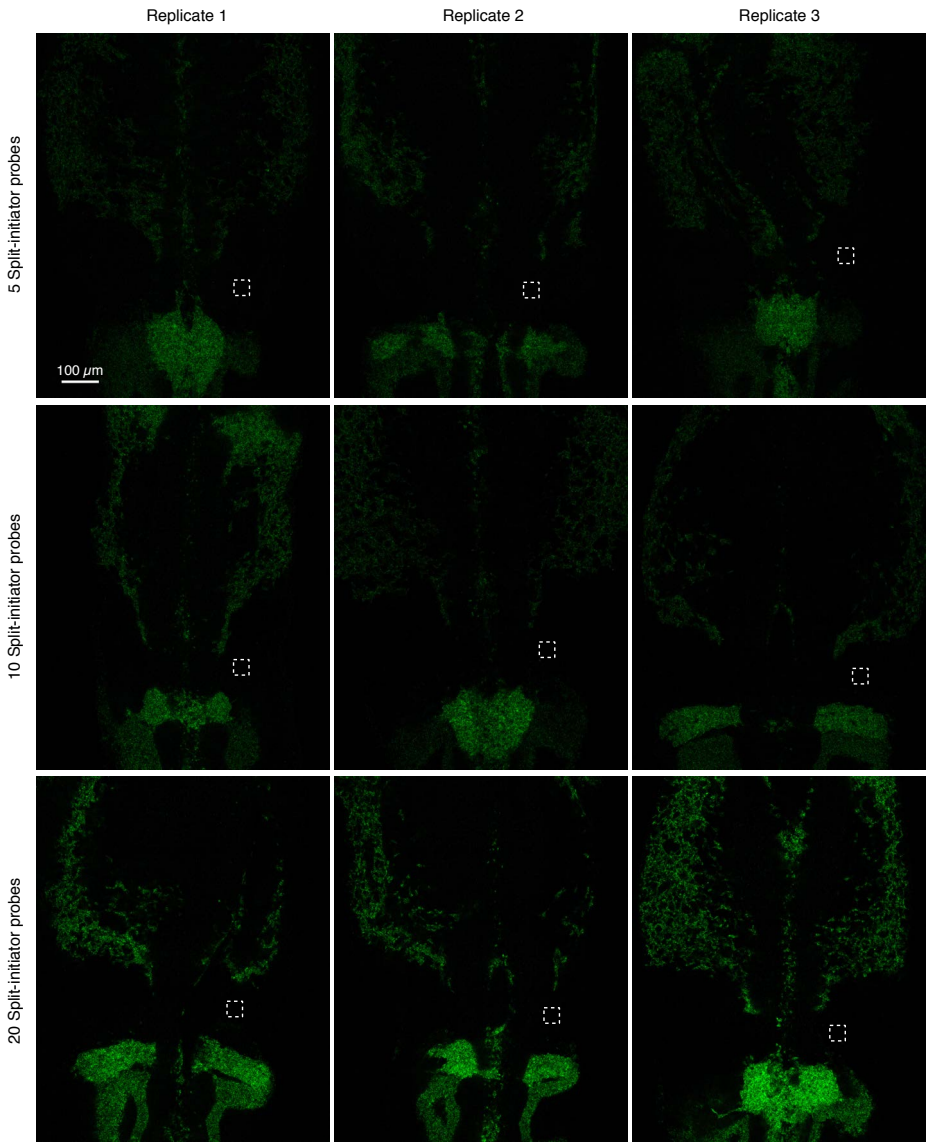

Figure S6. Measurement of background for unoptimized split-initiator probe sets as a function of probe set size (cf. Figure 3A). Confocal images collected with the microscope PMT gain adjusted to avoid saturating pixels using 20 standard probes (Figure S5, row 3). Protocol: in situ HCR v3.0 (Section S2.1). Target mRNA: *Sox10*. Probe set: 5, 10, or 20 split-initiator probe pairs. Amplifier: B3-Alexa647. Whole-mount chicken embryos fixed stage HH 11.

Probe type	Probe set size	BACK
Standard (v2.0)	5	210 ± 30
	10	1160 ± 60
	20	1500 ± 100
Split-initiator (v3.0)	5	29 ± 1
	10	26 ± 3
	20	28 ± 1

Table S10. Estimated background for standard and split-initiator probes sets as a function of probe set size (cf. Figure 3A). Mean $\pm$ standard error, $N = 3$ replicate embryos. Analysis based on rectangular regions depicted in Figures S5 and S6 using methods of Section S1.4.2.

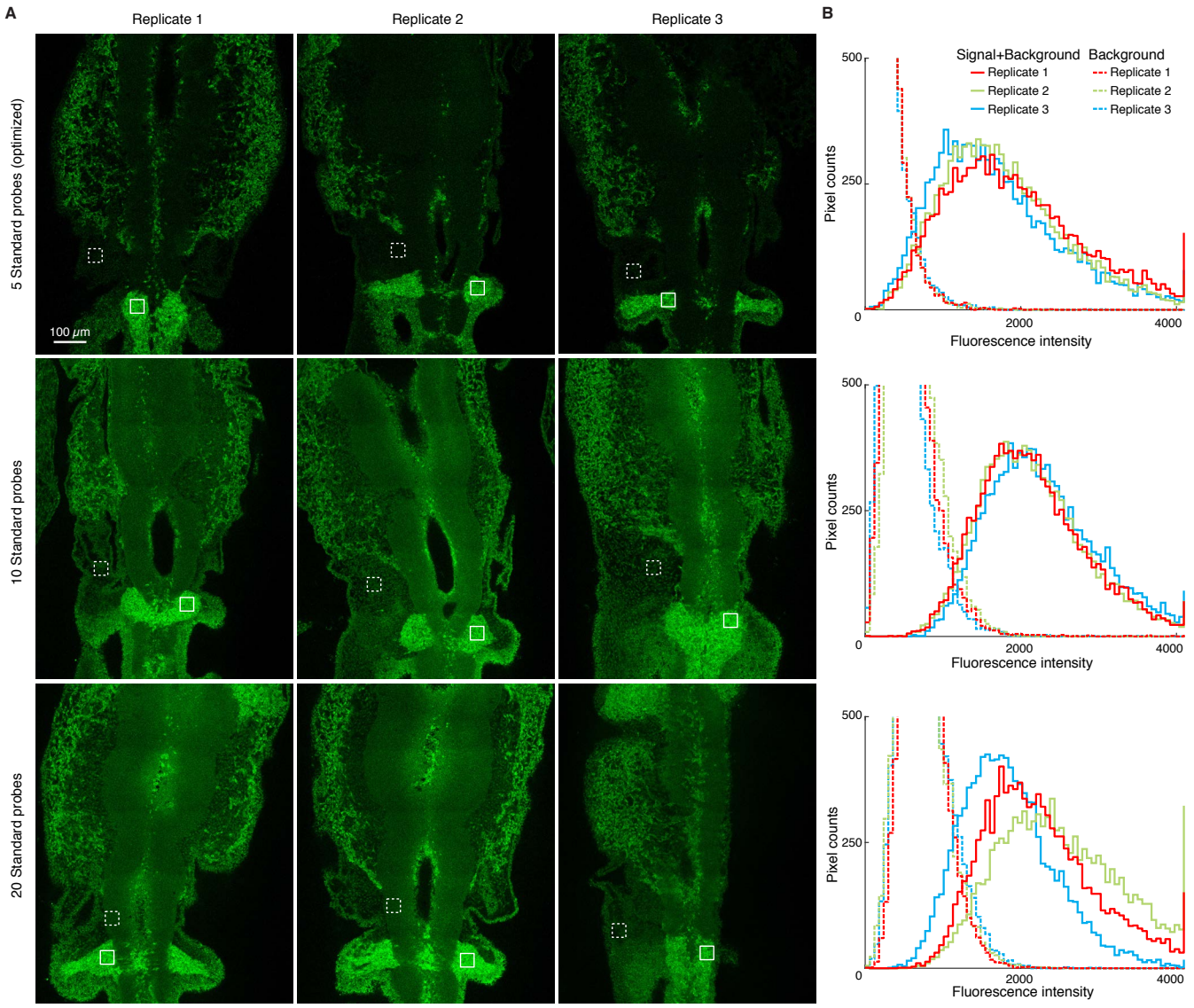

Figure S7. Measurement of signal and background for unoptimized standard probe sets as a function of probe set size (cf. Figure 3B). (A) Confocal images collected with the microscope PMT gain optimized for each probe set. (B) Pixel intensity histograms for Signal + Background (pixels within solid boundary) and Background (pixels within dashed boundary) per embryo in panel A. Protocol: in situ HCR v2.0 (Section S8 of Choi *et al.* (2016)). Target mRNA: *Sox10*. Probe set: 5, 10, or 20 standard probes. Amplifier: B3-Alexa647. Whole-mount chicken embryos fixed stage HH 11.

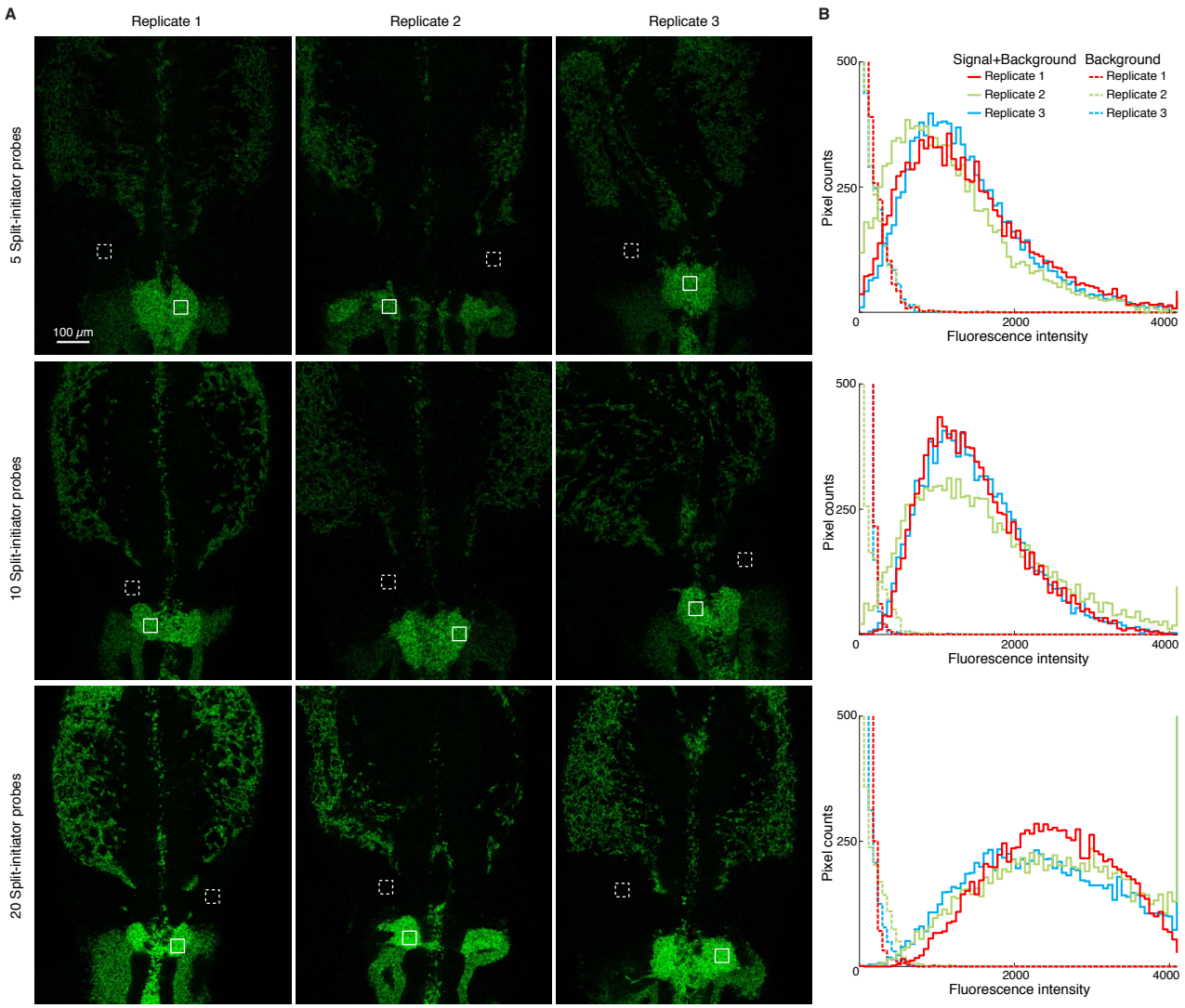

Figure S8. Measurement of signal and background for unoptimized split-initiator probe sets as a function of probe set size (cf. Figure 3B). (A) Confocal images collected with the microscope PMT gain optimized for each probe set. (B) Pixel intensity histograms for Signal + Background (pixels within solid boundary) and Background (pixels within dashed boundary) per embryo in panel A. Protocol: in situ HCR v3.0 (Section S2.1). Target mRNA: *Sox10*. Probe set: 5, 10, or 20 split-initiator probe pairs. Amplifier: B3-Alexa647. Whole-mount chicken embryos fixed stage HH 11.

Probe type	Probe set size	BACK	SIG+BACK	SIG	SIG/BACK
Standard (v2.0)	5	200.3 ± 1.1	1770 ± 70	1570 ± 70	7.8 ± 0.3
	10	540 ± 40	2170 ± 40	1630 ± 60	3.0 ± 0.2
	20	720 ± 10	2200 ± 200	1400 ± 200	2.0 ± 0.3
Split-initiator (v3.0)	5	43.7 ± 0.6	1270 ± 70	1230 ± 70	28.2 ± 1.7
	10	34.0 ± 1.2	1480 ± 30	1450 ± 30	42.5 ± 1.8
	20	37 ± 3	2460 ± 40	2420 ± 40	65 ± 5

Table S11. Estimated signal-to-background for standard and split-initiator probes sets as a function of probe set size (cf. Figure 3B). For this signal-to-background study, the microscope PMT gain was optimized for each probe set so raw background and signal values should only be compared within row. Mean $\pm$ standard error, $N = 3$ replicate embryos. Analysis based on rectangular regions depicted in Figures S7 and S8 using methods of Section S1.4.2.

S3.2.2 Measurement of background and signal-to-background for standard and split-initiator probes with identical target-binding domains

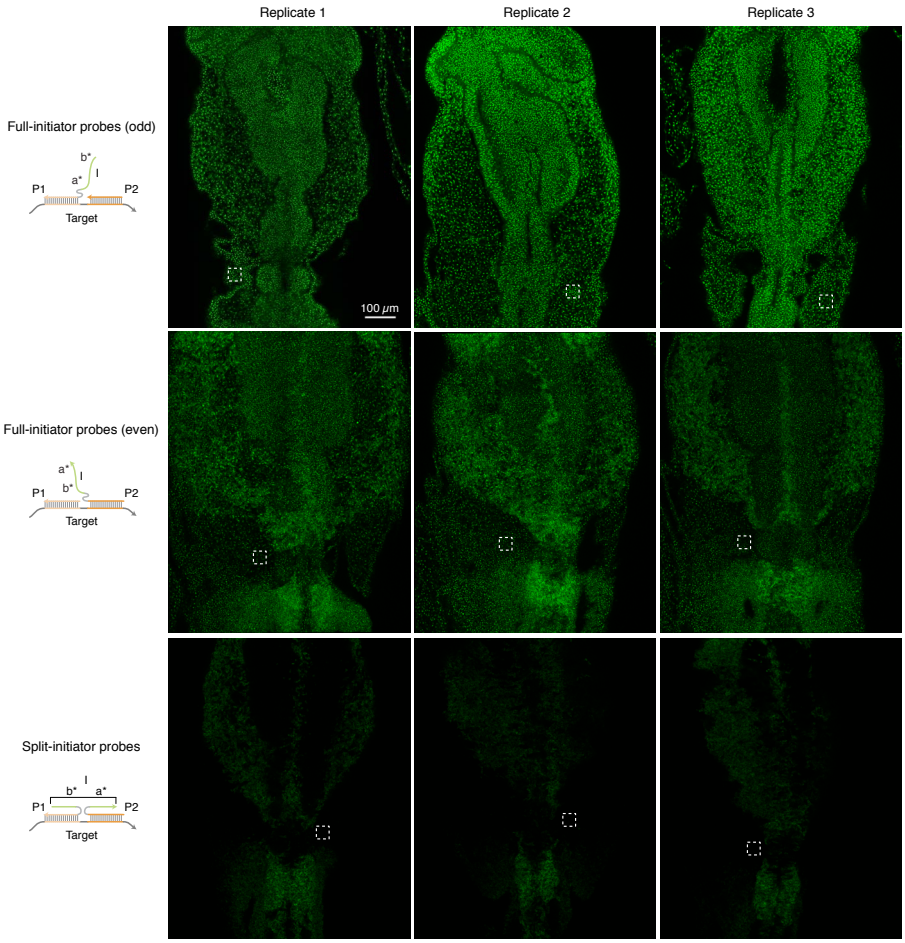

Figure S9. Measurement of background for standard and split-initiator probes with identical target-binding domains. (A) Confocal images collected with the microscope PMT gain adjusted to avoid saturating pixels using full-initiator odd probe sets (top row). Protocol: in situ HCR v3.0 (Section S2.1). Target mRNA: *Sox10*. Probe sets (20 probe pairs per probe set): full-initiator odd probes + initiator-free even probes (top row), initiator-free odd probes + full-initiator even probes (middle row), split-initiator odd and even probes (bottom row). Amplifier: B3-Alexa647. Whole-mount chicken embryos fixed stage HH 10.

Sample	BACK
Full-initiator probes (odd)	910 ± 80
Full-initiator probes (even)	330 ± 40
Split-initiator probes	19.9 ± 0.9

Table S12. Estimated background for standard and split-initiator probes with identical target-binding domains. Mean $\pm$ standard error, $N = 3$ replicate embryos. Analysis based on rectangular regions depicted in Figure S9 using methods of Section S1.4.2.

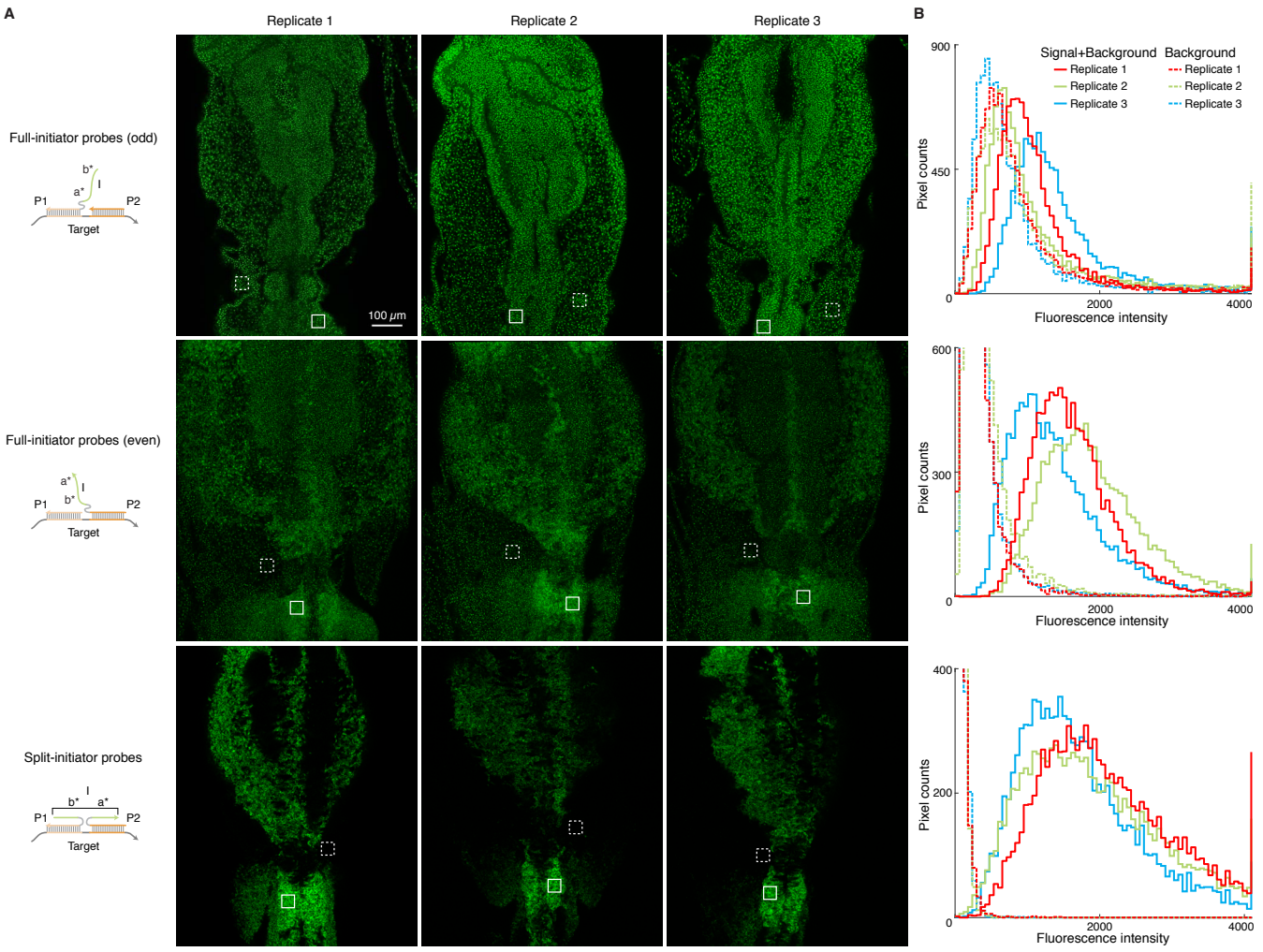

Figure S10. Measurement of signal and background for standard and split-initiator probes with identical target-binding domains. (A) Confocal images collected with the microscope PMT gain optimized for each probe set. (B) Pixel intensity histograms for Signal + Background (pixels within solid boundary) and Background (pixels within dashed boundary) per embryo in panel A. Protocol: in situ HCR v3.0 (Section S2.1). Target mRNA: *Sox10*. Probe sets (20 probe pairs per probe set): full-initiator odd probes + initiator-free even probes (top row), initiator-free odd probes + full-initiator even probes (middle row), split-initiator odd and even probes (bottom row). Amplifier: B3-Alexa647. Whole-mount chicken embryos fixed stage HH 10.

Sample	BACK	SIG+BACK	SIG	SIG/BACK
Full-initiator probes (odd)	910 ± 80	1230 ± 110	320 ± 140	0.4 ± 0.2
Full-initiator probes (even)	330 ± 40	1610 ± 150	1280 ± 160	3.9 ± 0.6
Split-initiator probes	26.4 ± 0.7	1870 ± 110	1840 ± 110	70 ± 5

Table S13. Estimated signal-to-background for standard and split-initiator probes with identical target-binding domains. Mean $\pm$ standard error, $N = 3$ replicate embryos. Analysis based on rectangular regions depicted in Figure S10 using methods of Section S1.4.2.

S3.2.3 Measurement of signal, background, signal-to-background, background components, and split-initiator HCR suppression

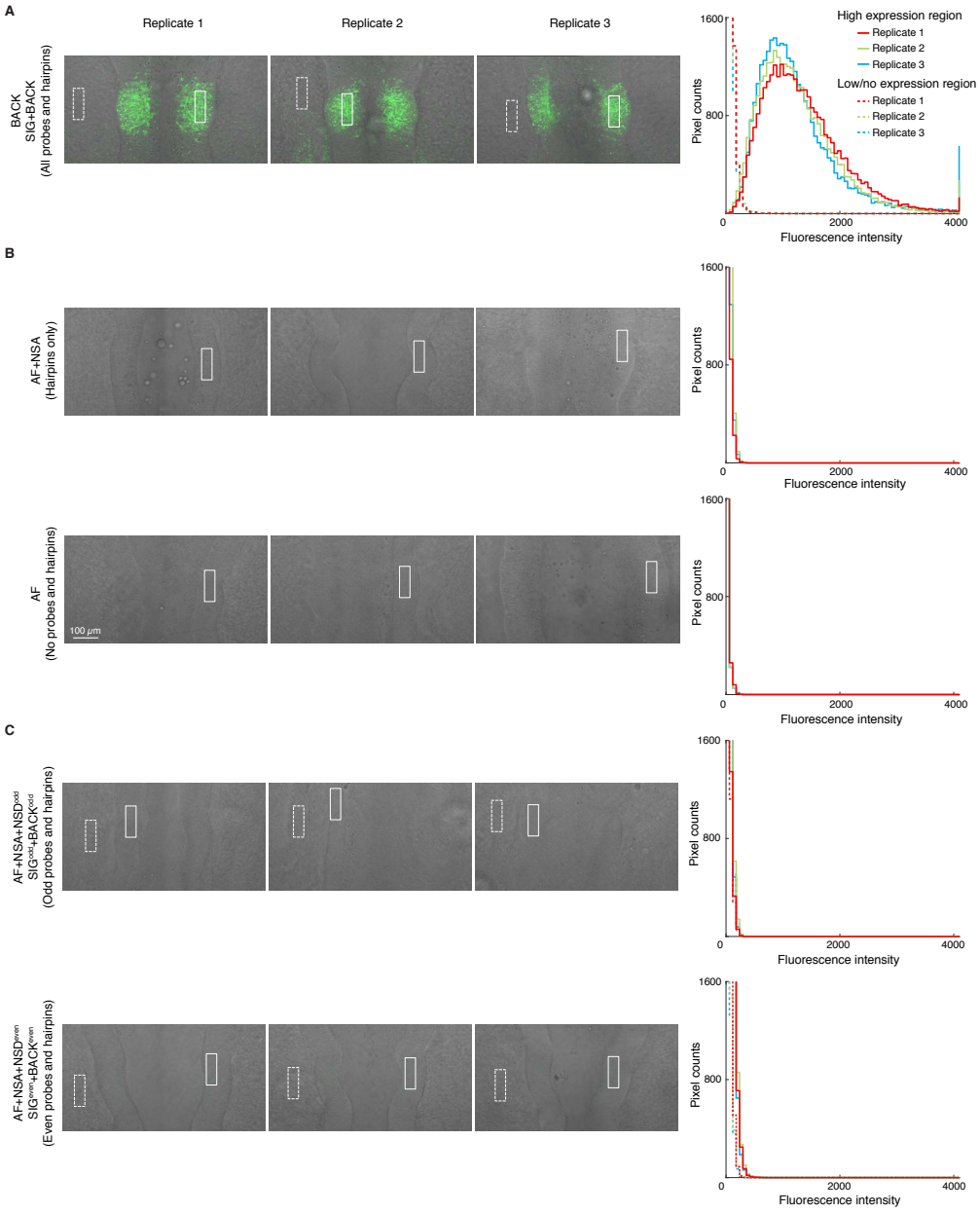

Figure S11. Measurement of signal and background, background components, and split-initiator HCR suppression. Confocal image: fluorescence merged with bright field. (A) Signal and background: use experiment of Type 1 in Table S5A (odd probes + even probes + hairpins) to measure SIG+BACK (region of high expression) and BACK (region of no/low expression). (B) Background components: use experiment of Type 2 in Table S5B (no probes, hairpins only) to measure NSA+AF (region of high expression); use experiment of Type 3 (no probes, no hairpins) to measure AF (region of high expression). (C) Split-initiator HCR suppression: use experiment of Type 4 in Table S5C (odd probes, hairpins) to measure SIG^{odd}+$BACK^{odd}$ (region of high expression) and $BACK^{odd}$ (region of no/low expression); use experiment of Type 5 in Table S5C (even probes, hairpins) to measure SIG^{even}+$BACK^{even}$ (region of high expression) and $BACK^{even}$ (region of no/low expression). Left: confocal images collected with the microscope PMT gain optimized to avoid saturating pixels using the full method (top row). Right: pixel intensity histograms for a region of high expression (pixels within solid boundary) and/or low/no expression (pixels within dashed boundary) per embryo. Protocol: in situ HCR v3.0 (Section S2.1). Target mRNA: *EphA4*. Probe set: 20 split-initiator probe pairs. Amplifier: B2-Alexa647. Whole-mount chicken embryos fixed stage HH 11.

		Channel		Reagents		Expression	
	Quantity	B2-Alexa647		Probes	Hairpins	region	Figure
A	SIG+NSD+NSA+AF = SIG+BACK	1260	$\pm$ 30	odd + even	✓	high	S11C
	NSD+NSA+AF = BACK	19	$\pm$ 1	odd + even	✓	low/no	S11C
	SIG	1240	$\pm$ 30				
	SIG/BACK	67	$\pm$ 4				
B	NSA+AF	8.1	$\pm$ 0.9		✓	high	S11B
	AF	6.97	$\pm$ 0.08			high	S11A
	NSA	1.2	$\pm$ 0.9				
	NSD	10.5	$\pm$ 1.3				
C	NSD^{odd}+NSA+AF = $BACK^{odd}$	11.0	$\pm$ 1.0	odd	✓	low/no	S11D
	NSD^{even}+NSA+AF = $BACK^{even}$	10.0	$\pm$ 0.6	even	✓	low/no	S11E
	SIG^{odd}+$BACK^{odd}$	11.5	$\pm$ 1.1	odd	✓	high	S11D
	SIG^{even}+$BACK^{even}$	31.8	$\pm$ 1.7	even	✓	high	S11E
	NSD^{odd}	2.8	$\pm$ 1.4				
	NSD^{even}	1.9	$\pm$ 1.1				
	SIG^{odd}	<1.5					
	SIG^{even}	21.8	$\pm$ 1.8				
	SIG/SIG^{odd}	>800					
	SIG/SIG^{even}	57	$\pm$ 5				

Table S14. Estimated signal-to-background, background components, and split-initiator HCR suppression. (A) Signal-to-background (SIG/BACK) based on methods of Section S1.4.2. (B) Background components (AF, NSA, NSD) based on methods of Section S1.4.3. (C) Split-initiator HCR suppression (SIG/SIG^{odd}, SIG/SIG^{even}) based on methods of Section S1.4.4. Mean $\pm$ standard error, $N = 3$ replicate embryos. Analysis based on rectangular regions depicted in Figure S11.

S3.3 Multiplexed 4-channel mRNA imaging with high signal-to-background in whole-mount chicken embryos (cf. Figure 4)

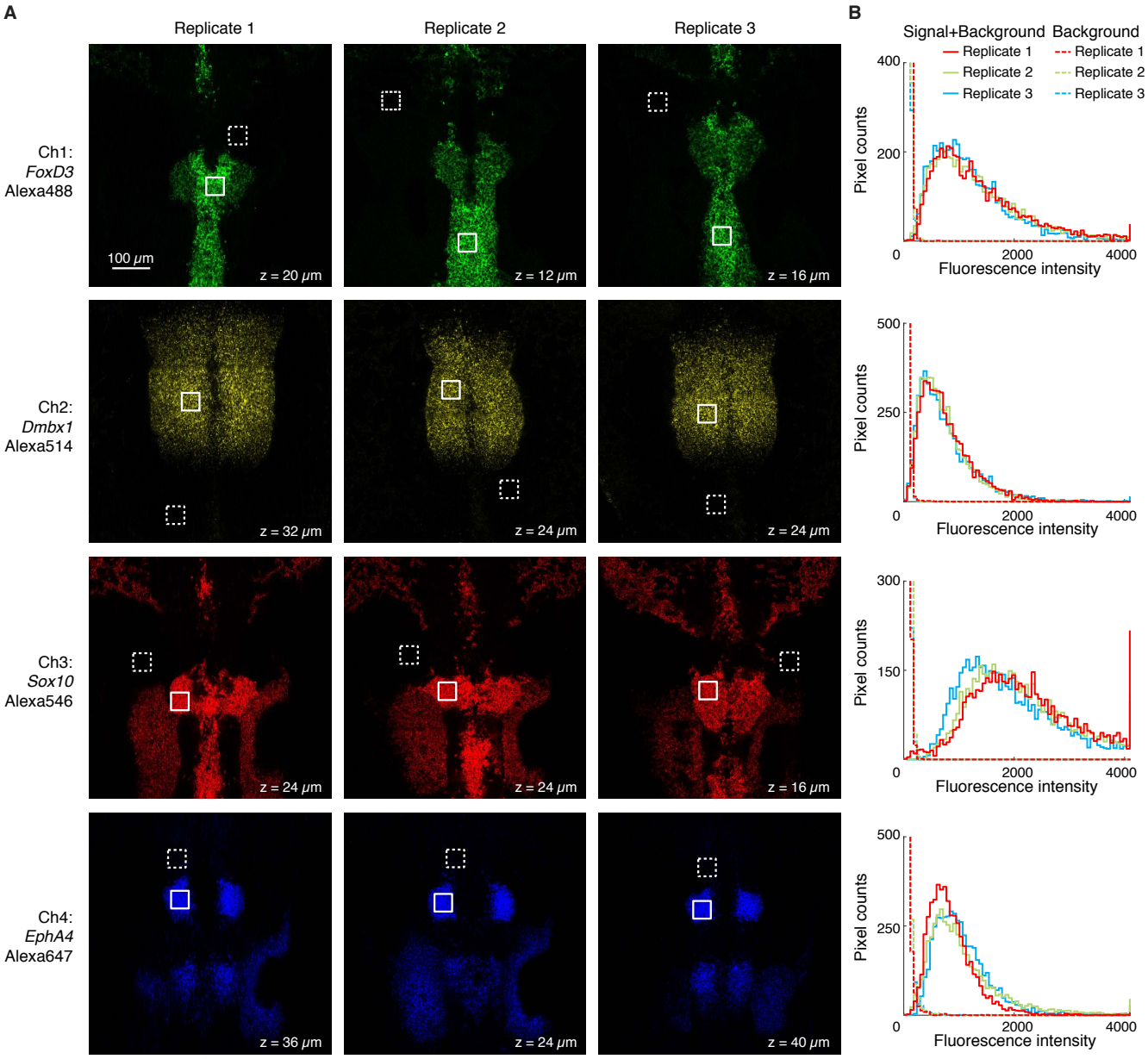

Figure S12. Measurement of signal and background for multiplexed 4-channel mRNA imaging (cf. Figure 4). (A) Individual channels of 4-channel confocal images. For each of three replicate embryos, a representative optical section was selected for each channel based on the expression depth of the corresponding target mRNA. (B) Pixel intensity histograms for Signal + Background (pixels within solid boundary) and Background (pixels within dashed boundary). Ch1: target mRNA *FoxD3*, probe set with 12 split-initiator probe pairs, amplifier B4-Alexa488. Ch2: target mRNA *Dmbx1*, probe set with 20 split-initiator probe pairs, amplifier B1-Alexa514. Ch 3: target mRNA *Sox10*, probe set with 20 split-initiator probe pairs, amplifier B3-Alexa546. Ch4: *EphA4*, probe set with 20 split-initiator probe pairs, amplifier B2-Alexa647. Whole-mount chicken embryos fixed stage HH 10.

Target mRNA	BACK	SIG+BACK	SIG	SIG/BACK
FoxD3	36 ± 3	1270 ± 50	1230 ± 50	34 ± 3
Dmbx1	16.4 ± 0.2	730 ± 20	710 ± 20	43.4 ± 1.0
Sox10	34.0 ± 0.2	2030 ± 80	2000 ± 80	59 ± 3
EphA4	35 ± 3	980 ± 70	950 ± 70	27 ± 3

Table S15. Estimated signal-to-background for multiplexed 4-channel mRNA imaging. Mean $\pm$ standard error, $N = 3$ replicate embryos. Analysis based on rectangular regions depicted in Figure S12 using methods of Section S1.4.2.

S3.4 qHCR imaging: analog mRNA relative quantitation with subcellular resolution in whole-mount chicken embryos (cf. Figure 5)

S3.4.1 Testing for a crowding effect

In order to perform multiplexed quantitative imaging using HCR, it is important that there is not a crowding effect in which amplification polymers tethered to one target molecule affect the signal intensity for a different target molecule. To test for a possible crowding effect, we imaged two target mRNAs that are highly expressed in the same cells (*EphA4* and *Egr2*) individually (1-target studies) and also simultaneously (2-target studies) within whole-mount chicken embryos. Figure S13 compares the signal intensity distributions for 1-target and 2-target studies, revealing similar intensity distributions whether targets were detected alone or together, suggesting that there is not a significant crowding effect (either antagonistic or synergistic).

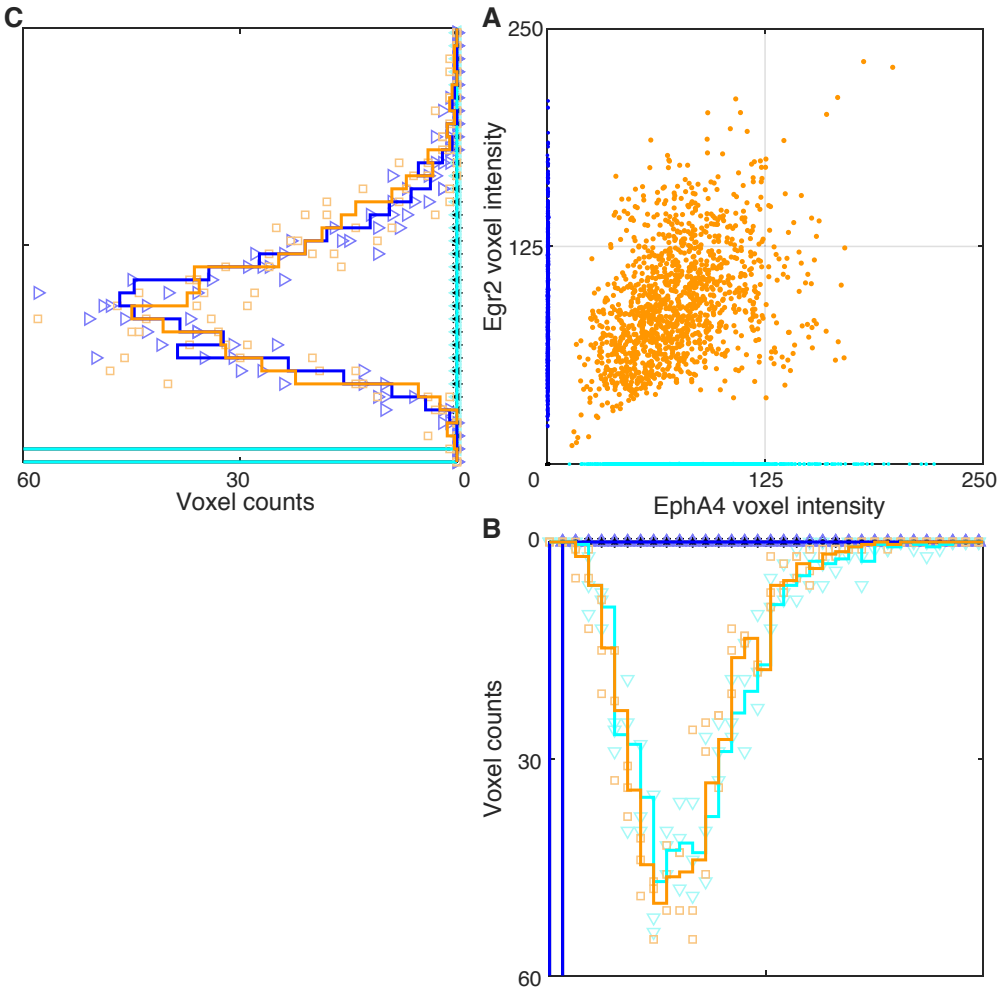

Figure S13. Comparison of signal intensity distributions for individual and simultaneous imaging of *EphA4* and *Egr2*. (A) Raw voxel intensity scatter plot: *Egr2* channel vs *EphA4* channel. (B) Raw voxel intensity histogram for *EphA4* channel. (C) Raw voxel intensity histogram for *Egr2* channel. In panels B and C, solid lines denote average histograms over 3 replicate embryos while symbols denote individual histograms (1 histogram per replicate). Orange data: signal plus background for *EphA4* and *Egr2* (Figure S14). Cyan data: signal plus background for *EphA4* and background for *Egr2* (Figure S15). Blue data: background for *EphA4* and signal plus background for *Egr2* (Figure S16). Black data (near origin): background for *EphA4* and *Egr2* (Figure S17). Voxel size: 2.1×2.1×2.7 μm. Whole-mount wildtype chicken embryos fixed stage HH 10.

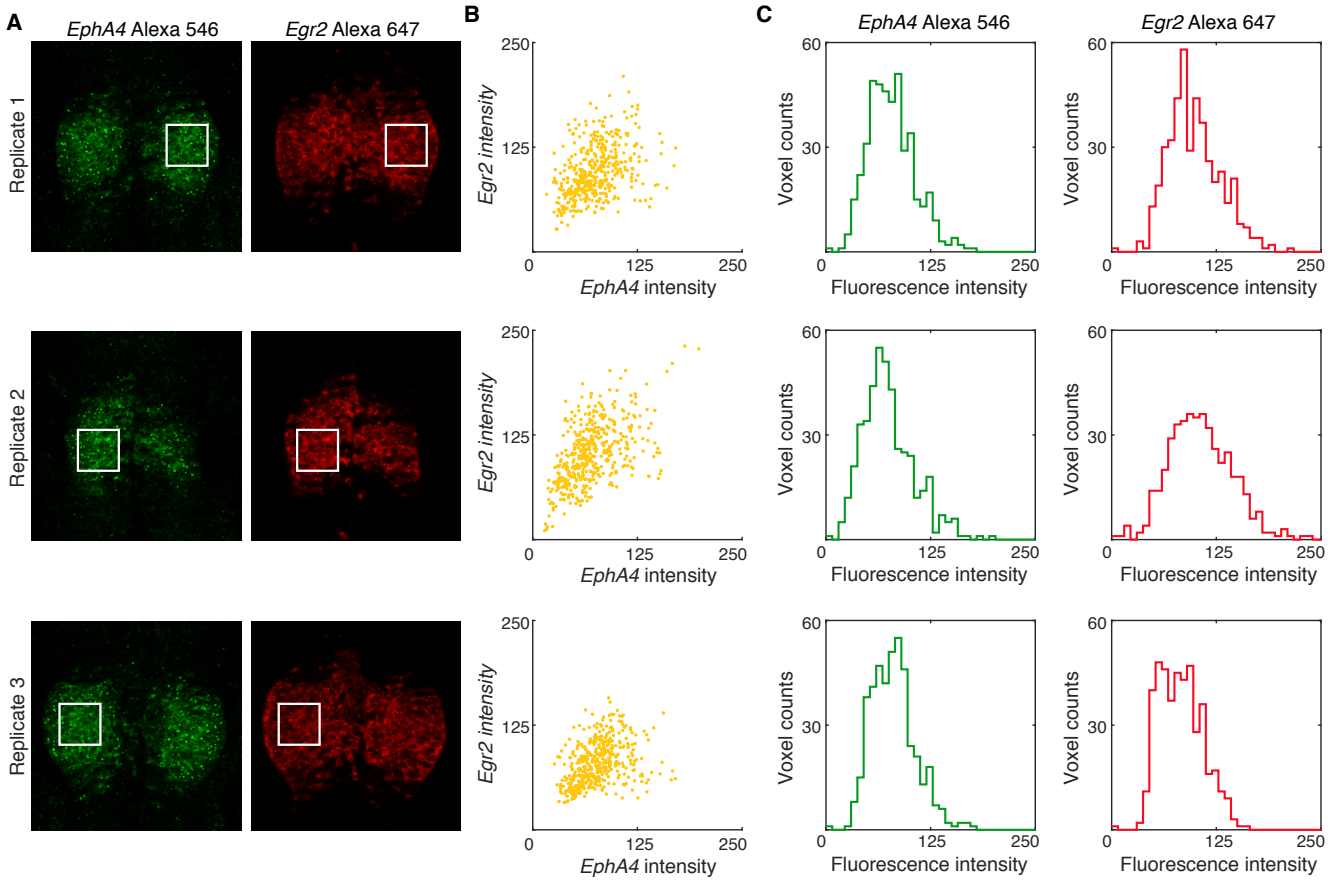

Figure S14. Characterizing signal plus background for *EphA4* and *Egr2* in a 2-target experiment. (A) Individual channels from 2-channel confocal images depicting regions used to estimate signal plus background. *EphA4* channel: 20 probe pairs and amplifier B1-Alexa546. *Egr2* channel: 20 probe pairs and amplifier B3-Alexa647. For each of 3 replicate embryos, a representative optical section was selected based on the expression depth of the target mRNAs. Pixel size: 0.4×0.4 μm. (B) Raw voxel intensity scatter plots for the selected regions of panel A representing signal plus background for *EphA4* and *Egr2*. (C) Raw voxel intensity histograms for the selected regions of panel A representing signal plus background for *EphA4* and *Egr2*. Same microscope settings used for all replicates in Figures S14–S17. Voxel size: 2.1×2.1×2.7 μm. Whole-mount wildtype chicken embryos fixed stage HH 10.

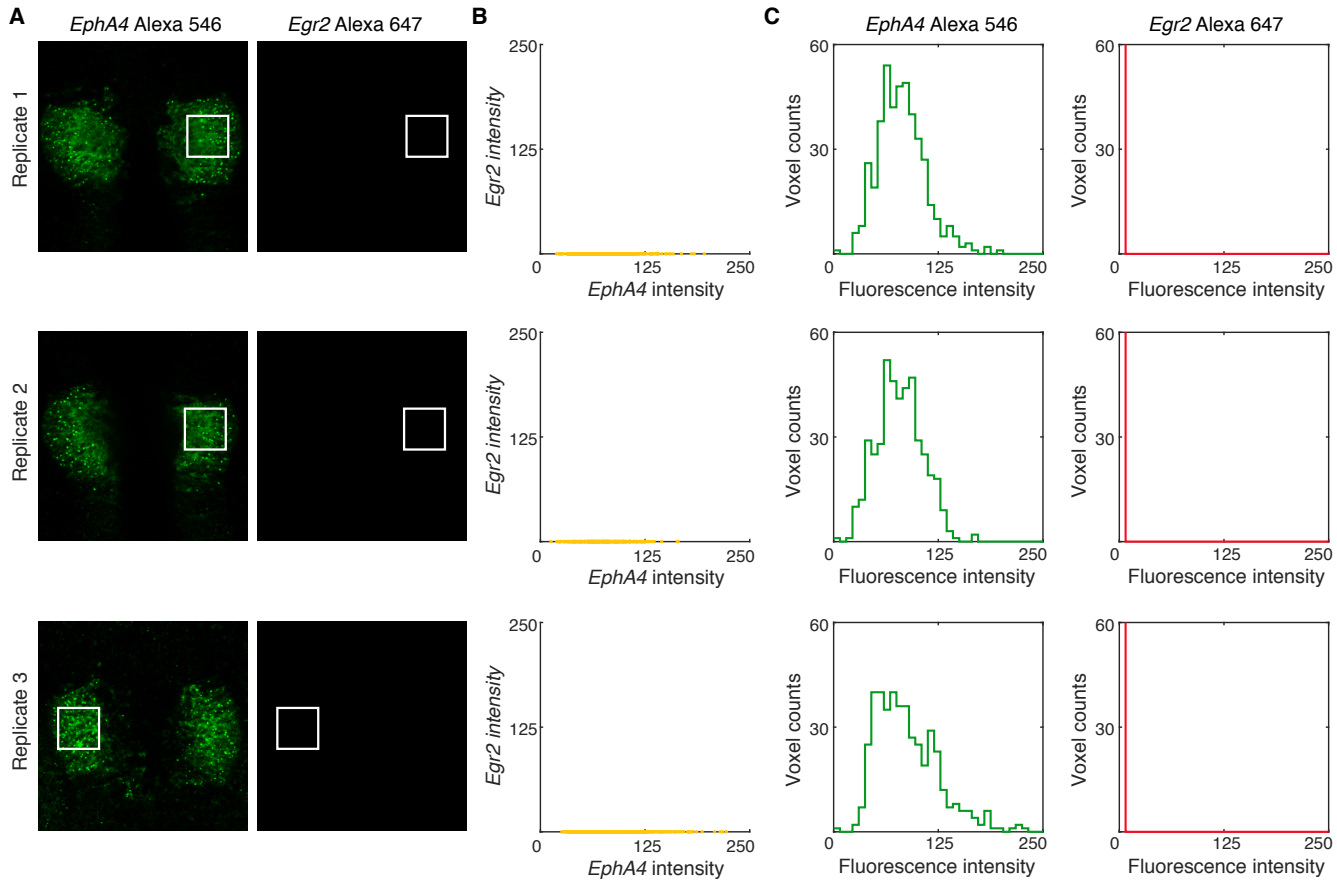

Figure S15. Characterizing signal plus background for *EphA4* in a 1-target experiment. (A) Individual channels from 2-channel confocal images depicting regions used to estimate signal plus background for *EphA4* and background for *Egr2*. *EphA4* channel: 20 probe pairs and amplifier B1-Alexa546. *Egr2* channel: no probes, no amplifier. For each of 3 replicate embryos, a representative optical section was selected based on the expression depth of the target mRNA. Pixel size: 0.4×0.4 μm. (B) Raw voxel intensity scatter plots for the selected regions of panel A representing signal plus background for *EphA4* and background for *Egr2*. (C) Raw voxel intensity histograms for the selected regions of panel A representing signal plus background for *EphA4* and background for *Egr2*. Same microscope settings used for all replicates in Figures S14–S17. Voxel size: 2.1×2.1×2.7 μm. Whole-mount wildtype chicken embryos fixed stage HH 10.

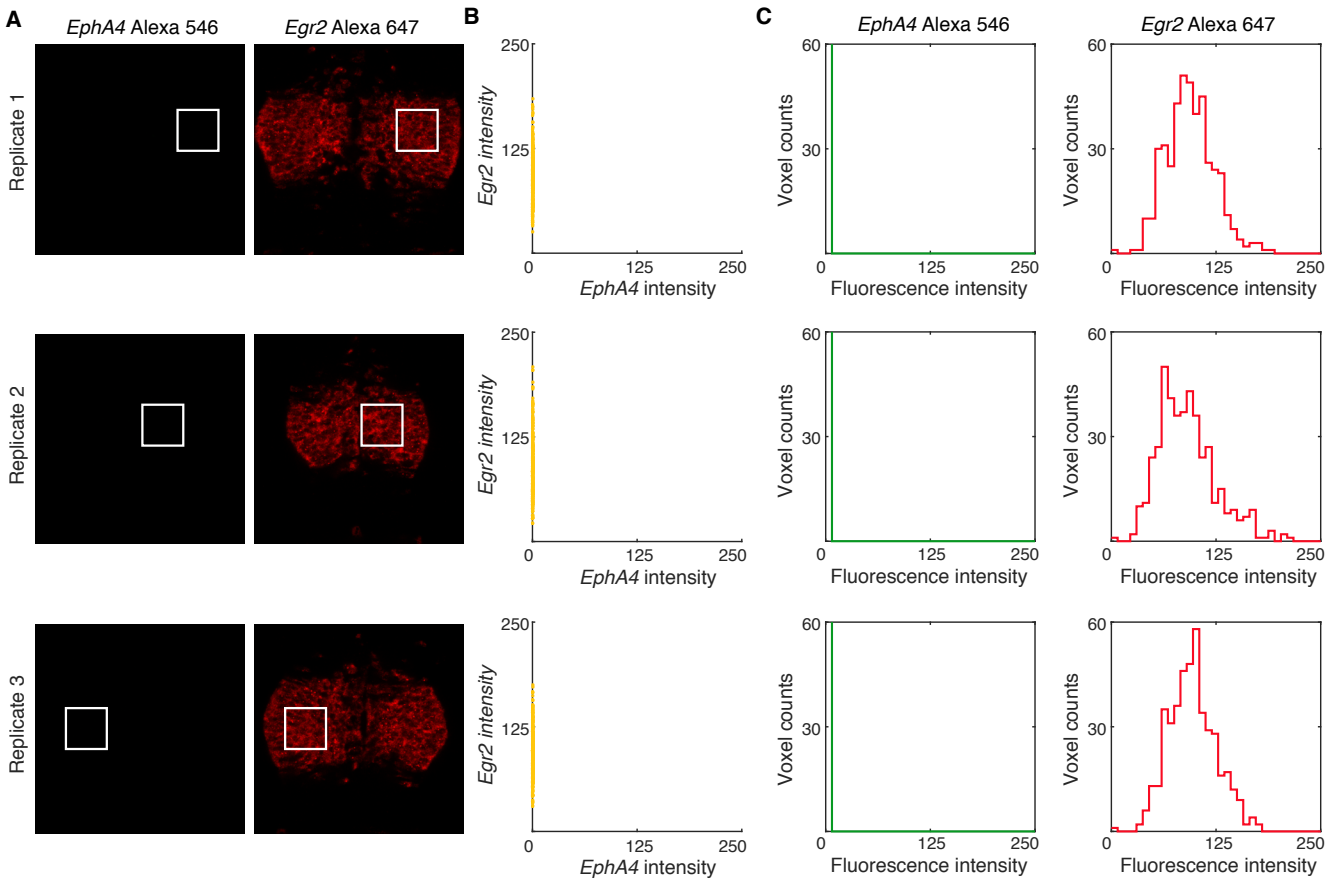

Figure S16. Characterizing signal plus background for *Egr2* in a 1-target experiment. (A) Individual channels from 2-channel confocal images depicting regions used to estimate background for *EphA4* and signal plus background for *Egr2*. *EphA4* channel: no probes, no amplifier. *Egr2* channel: 20 probe pairs and amplifier B3-Alexa647. For each of 3 replicate embryos, a representative optical section was selected based on the expression depth of the target mRNA. Pixel size: 0.4×0.4 μm. (B) Raw voxel intensity scatter plots for the selected regions of panel A representing background for *EphA4* and signal plus background for *Egr2*. (C) Raw voxel intensity histograms for the selected regions of panel A representing background for *EphA4* and signal plus background for *Egr2*. Same microscope settings used for all replicates in Figures S14–S17. Voxel size: 2.1×2.1×2.7 μm. Whole-mount wildtype chicken embryos fixed stage HH 10.

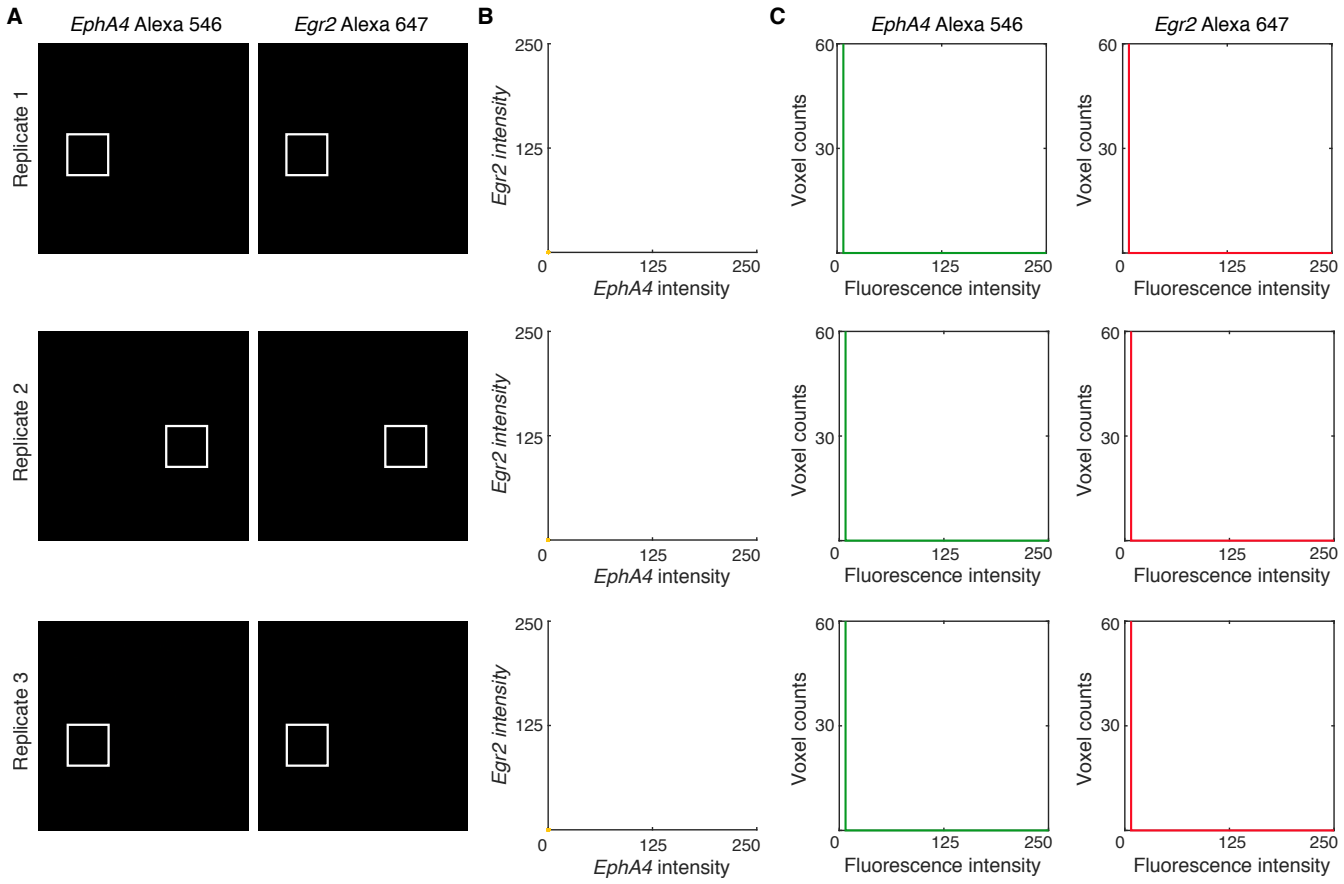

Figure S17. Characterizing background for *EphA4* and *Egr2*. Individual channels from 2-channel confocal images depicting regions used to estimate background using the standard HCR v3.0 in situ protocol (Section S2.1) omitting probes (BACK $\approx$ AF + NSA; see Section S1.4 for definitions). For each of 3 replicate embryos, a representative optical section was selected at approximately the depth where *EphA4* and *Egr2* are expressed. Same microscope settings used for all replicates in Figures S14–S17. Pixel size: 0.4×0.4 μm. (B) Raw voxel intensity scatter plots for the selected region of panel A. (C) Raw voxel intensity histograms for the scatter plots of panel B. Voxel size: 2.1×2.1×2.7 μm. Whole-mount wildtype chicken embryos fixed stage HH 10.

S3.4.2 Redundant 2-channel detection of *Dmbx1*

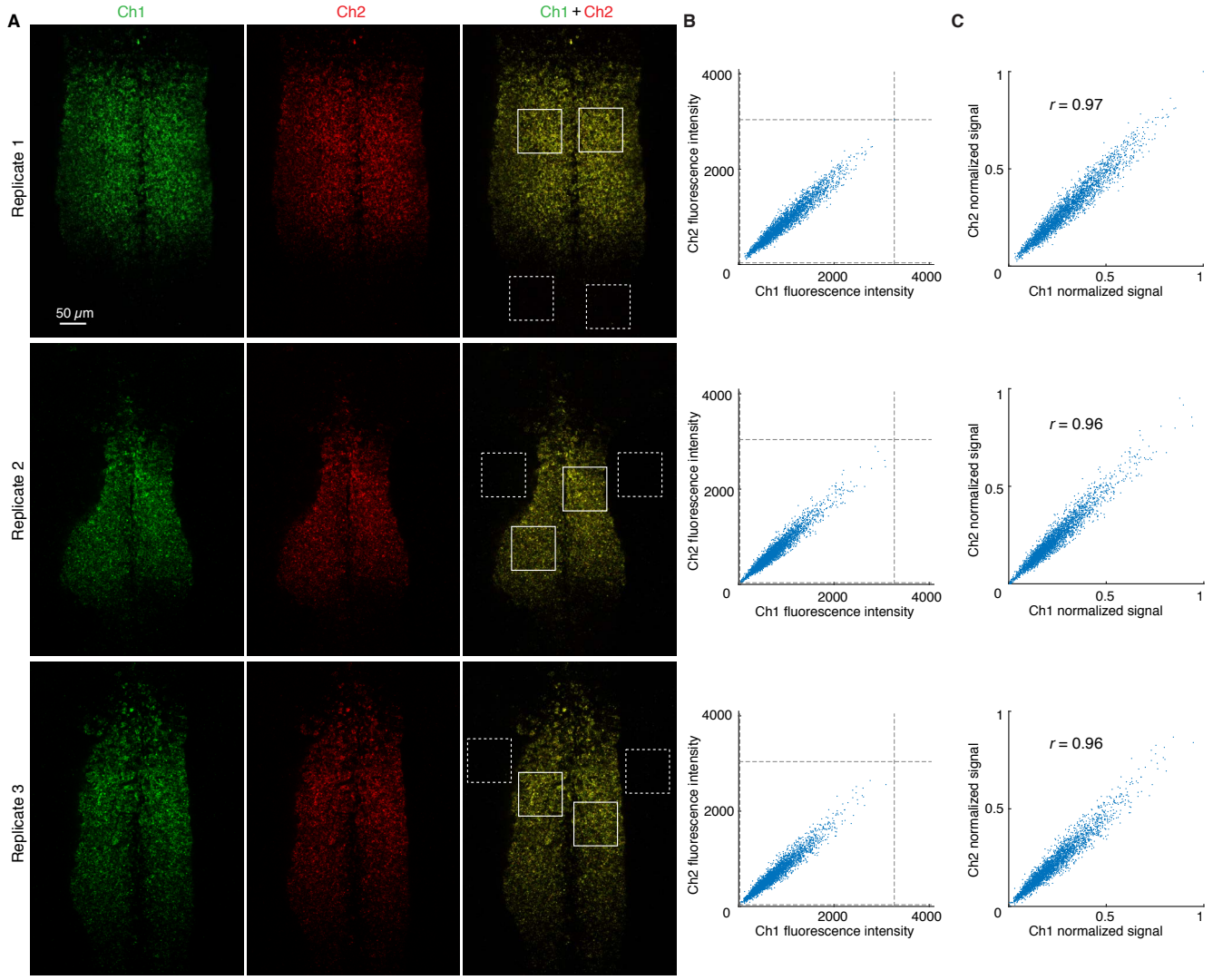

Figure S18. Redundant 2-channel detection of *Dmbx1*. (A) Confocal images: individual channels and merge. Solid boundaries denote regions of high expression; dashed boundaries denote regions of no/low expression. Pixel size: 0.2×0.2 μm. Probe sets: 20 split-initiator probe pairs per channel. Amplifiers: B1-Alexa546 (Ch1) and B2-Alexa647 (Ch2). Whole-mount chicken embryos fixed stage HH 10. (B) Raw voxel intensity scatter plots representing signal plus background for voxels within solid boundaries of panel A. Voxel size: 2.1×2.1×2.7 μm. Dashed lines represent BOT and TOP values (Table S16) used to normalize data for panel C using methods of Section S1.4.5. (C) Normalized voxel intensity scatter plots representing estimated normalized signal (Pearson correlation coefficient, r).

S3.4.3 Redundant 2-channel detection of *EphA4*

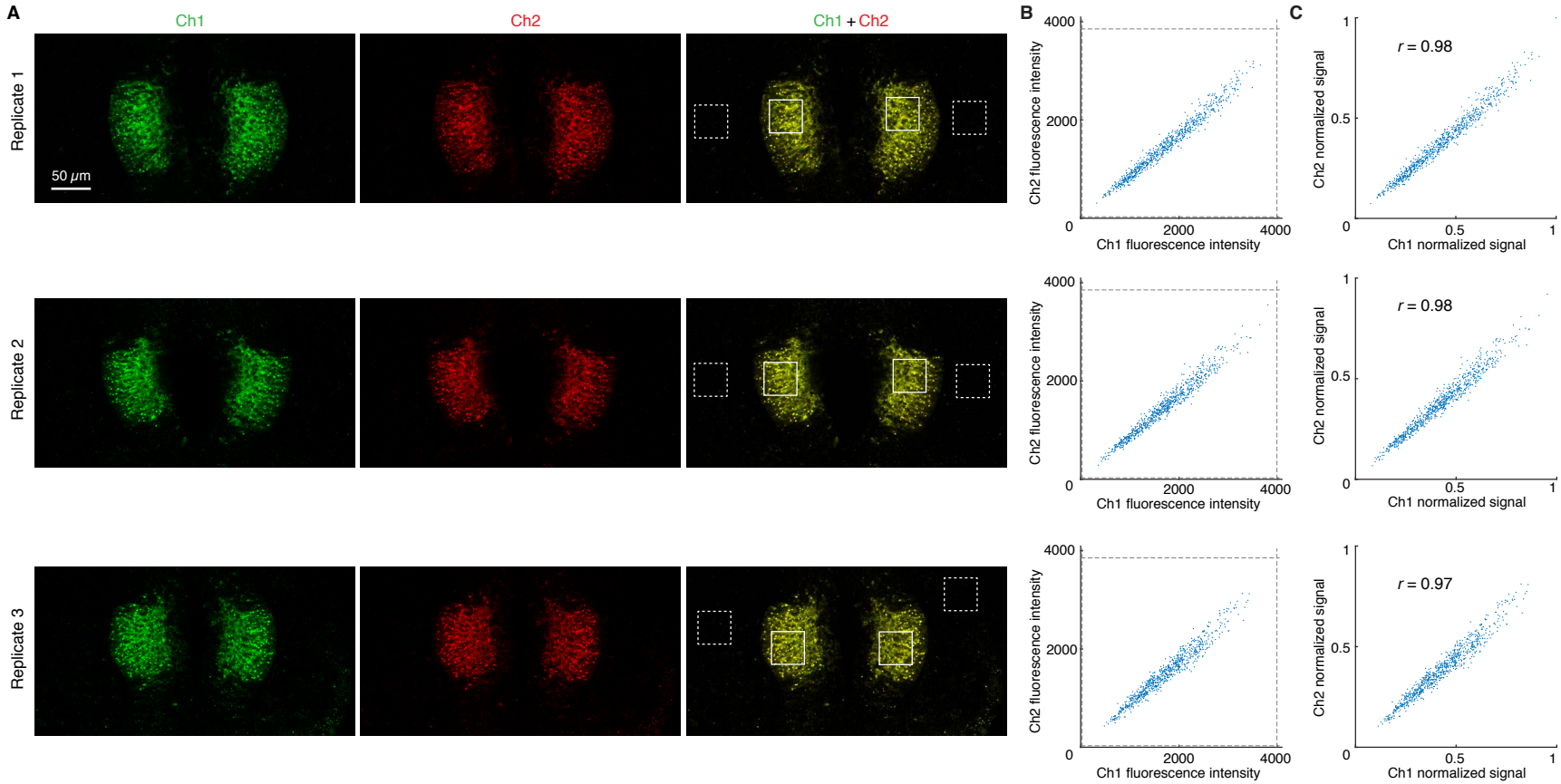

Figure S19. Redundant 2-channel detection of *EphA4* (cf. Figure 5). (A) Confocal images: individual channels and merge. Solid boundaries denote regions of high expression; dashed boundaries denote regions of no/low expression. Pixel size: 0.2×0.2 μm. Probe sets: 20 split-initiator probe pairs per channel. Amplifiers: B1-Alexa546 (Ch1) and B2-Alexa647 (Ch2). Whole-mount chicken embryos fixed stage HH 10. (B) Raw voxel intensity scatter plots representing signal plus background for voxels within solid boundaries of panel A. Voxel size: 2.1×2.1×2.7 μm. Dashed lines represent BOT and TOP values (Table S16) used to normalize data for panel C using methods of Section S1.4.5. (C) Normalized voxel intensity scatter plots representing estimated normalized signal (Pearson correlation coefficient, r).

Target mRNA	Channel	BACK	SIG+BACK	SIG	SIG/BACK	BOT	TOP
Dmbx1	Alexa546	23.9 ± 1.1	840 ± 80	810 ± 80	34 ± 4	24	3266
Dmbx1	Alexa647	35 ± 5	770 ± 70	730 ± 70	21 ± 3	35	3040
EphA4	Alexa546	28.7 ± 1.1	1720 ± 10	1693 ± 13	59 ± 2	29	3995
EphA4	Alexa647	29.7 ± 1.1	1490 ± 10	1455 ± 10	48.9 ± 1.8	30	3855

Table S16. Estimated signal-to-background for redundant 2-channel detection of *Dmbx1* and *EphA4*. Mean $\pm$ standard error, $N = 3$ replicate embryos. Analysis based on rectangular regions depicted in Figure S18A and S19A using methods of Section S1.4.2. BOT and TOP values used to calculate normalized voxel intensities for scatter plots of Figures 5C, S18C, and S19C using methods of Section S1.4.5.

S3.5 In situ validation of automatic background suppression with split-initiator probes for mRNA flow cytometry with cultured human and bacterial cells

The methods of Sections S1.5.2 and S1.5.3 are used to measure:

- signal, background, and signal-to-background (Figures S20A–S22A and Tables S17A–S19A) .
- background components (AF, NSA, NSD; Figures S20A–S22A and Tables S17B–S19B).
- split-initiator HCR suppression (Figures S20B–S22B and Tables S17C–S19C).

Additional measurements of these quantities are provided in the 2-channel experiments of Figures S24–S28 and Tables S20–S24.

S3.5.1 Measurement of signal, background, signal-to-background, background components, and split-initiator HCR suppression for *d2eGFP* transgenic target in HEK cells

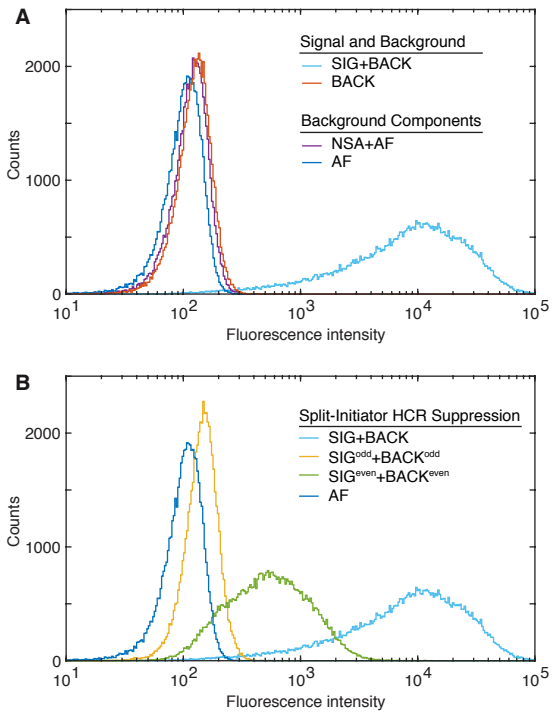

Figure S20. Measurement of signal and background, background components, and split-initiator HCR suppression for *d2eGFP* transgenic target in HEK cells (cf. Figure 6A). (A) Signal and background: use experiments of Types 1a and 1b in Table S7A (odd + even probes, hairpins) to measure SIG+BACK (GFP+ cells) and BACK (WT cells). Background components: use experiment of Type 2 in Table S7B (no probes, with hairpins) to measure NSA+AF (GFP+ cells); use experiment of Type 3 (no probes, no hairpins) to measure AF (GFP+ cells). (B) Split-initiator HCR suppression: use experiment of Types 4a in Table S7C (odd probes, hairpins) to measure SIG^{odd}+BACK^{odd} (GFP+ cells); use experiment of Type 5a in Table S7C (even probes, hairpins) to measure SIG^{even}+BACK^{even} (GFP+ cells). Distribution of single-cell fluorescence intensities. Protocol: in situ HCR v3.0 (Section S2.3). Probe set: 12 split-initiator probe pairs. Amplifier: B3-Alexa594. Sample: 55,000 HEK cells in suspension (GFP+ or WT).

		Channel	Reagents		
	Quantity	B3-Alexa594	Probes	Hairpins	Cell type
A	SIG+NSD+NSA+AF = SIG+BACK	13 220 ± 60	odd + even	✓	GFP+
	NSD+NSA+AF = BACK	128.5 ± 0.2	odd + even	✓	WT
	SIG	13 090 ± 60			
	SIG/BACK	101.8 ± 0.5			
B	NSA+AF	120.7 ± 0.5		✓	GFP+
	AF	104.7 ± 0.2		✓	GFP+
	NSA	16.0 ± 0.5			
	NSD	7.8 ± 0.5			
C	SIG^{odd}+$BACK^{odd}$	149.1 ± 0.3	odd	✓	GFP+
	SIG^{even}+$BACK^{even}$	710 ± 3	even	✓	GFP+
	SIG^{odd}	28.4 ± 0.6			
	SIG^{even}	589 ± 3			
	SIG/SIG^{odd}	461 ± 9			
	SIG/SIG^{even}	22.2 ± 0.1			

Table S17. Estimated signal-to-background, background components, and split-initiator HCR suppression for *d2eGFP* transgenic target in HEK cells (cf. Figure 6A). (A) Signal-to-background (SIG/BACK). (B) Background components (AF, NSA, NSD). (C) Split-initiator HCR suppression (SIG/SIG^{odd}, SIG/SIG^{even}). The signal estimates SIG^{odd} and SIG^{even} are calculated using the background approximation $BACK^{odd}$ = $BACK^{even}$ ≈ NSA+AF, which leads to lower bounds on SIG/SIG^{odd} and SIG/SIG^{even}. Mean ± standard error, $N = 55,000$ cells. Analysis based on single-cell intensities of Figure S20 using methods of Section S1.5.2.

S3.5.2 Measurement of signal, background, signal-to-background, background components, and split-initiator HCR suppression for *GAPDH* endogenous target in HEK cells

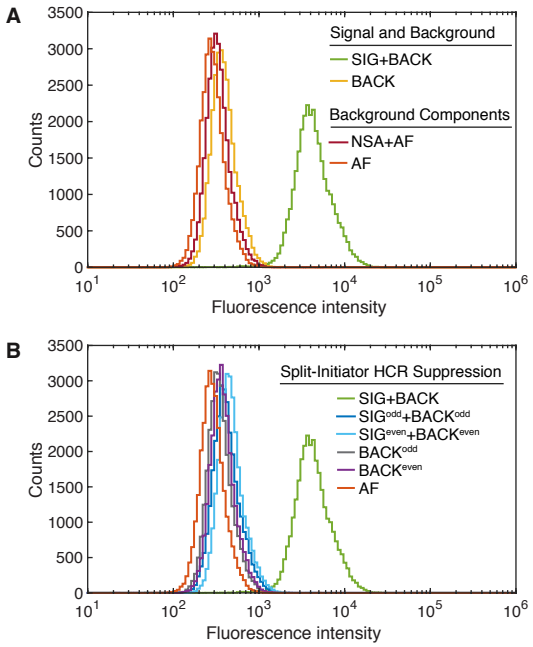

Figure S21. Measurement of signal and background, background components, and split-initiator HCR suppression for *GAPDH* endogenous target in HEK cells. (A) Signal and background: use experiments of Types 1a and 1b in Table S8A to measure SIG+BACK (even + odd probes, hairpins) and BACK (Tg(odd) + Tg(even) probes, hairpins). Background components: use experiment of Type 2 in Table S8B (no probes, with hairpins) to measure NSA+AF; use experiment of Type 3 (no probes, no hairpins) to measure AF. (B) Split-initiator HCR suppression: use experiment of Types 4a and 4b in Table S8C to measure SIG^{odd}+$BACK^{odd}$ (odd probes, hairpins) and $BACK^{odd}$ (Tg(odd) probes, hairpins); use experiments of Types 5a and 5b in Table S8C to measure SIG^{even}+$BACK^{even}$ (even probes, hairpins) and $BACK^{even}$ (Tg(even) probes, hairpins). Distribution of single-cell fluorescence intensities. Protocol: in situ HCR v3.0 (Section S2.3). Probe set: 10 split-initiator probe pairs. Amplifier: B4-Alexa594. Sample: 30,000 HEK cells in suspension (WT).

		Channel	Reagents		
	Quantity	B4-Alexa594	Probes	Hairpins	Cell type
A	SIG+NSD+NSA+AF = SIG+BACK	4775 ± 15	odd + even	✓	WT
	NSD+NSA+AF = BACK	414.0 ± 0.9	Tg(odd) + Tg(even)	✓	WT
	SIG	4362 ± 15			
	SIG/BACK	10.55 ± 0.04			
B	NSA+AF	353.7 ± 0.8		✓	WT
	AF	304.0 ± 0.7		✓	WT
	NSA	50 ± 1			
	NSD	60 ± 1			
C	SIG^{odd}+NSD^{odd}+NSA+AF = SIG^{odd}+$BACK^{odd}$	450 ± 7	odd	✓	WT
	NSD^{odd}+NSA+AF = $BACK^{odd}$	371.2 ± 0.8	Tg(odd)	✓	WT
	SIG^{even}+NSD^{even}+NSA+AF = SIG^{even}+$BACK^{even}$	499 ± 1	even	✓	WT
	NSD^{even}+NSA+AF = $BACK^{even}$	397.3 ± 0.8	Tg(even)	✓	WT
	NSD^{odd}	18 ± 1			
	NSD^{even}	44 ± 1			
	SIG^{odd}	79 ± 7			
	SIG^{even}	102 ± 1			
	SIG/SIG^{odd}	55 ± 5			
	SIG/SIG^{even}	42.9 ± 0.6			

Table S18. Estimated signal-to-background, background components, and split-initiator HCR suppression for *GAPDH* endogenous target in HEK cells. (A) Signal-to-background (SIG/BACK). (B) Background components (AF, NSA, NSD). (C) Split-initiator HCR suppression (SIG/SIG^{odd}, SIG/SIG^{even}). Mean ± standard error, $N = 30,000$ cells. Analysis based on single-cell intensities of Figure S21 using methods of Section S1.5.3.

S3.5.3 Measurement of signal, background, signal-to-background, background components, and split-initiator HCR suppression for *eGFP* transgenic target in *E. coli*

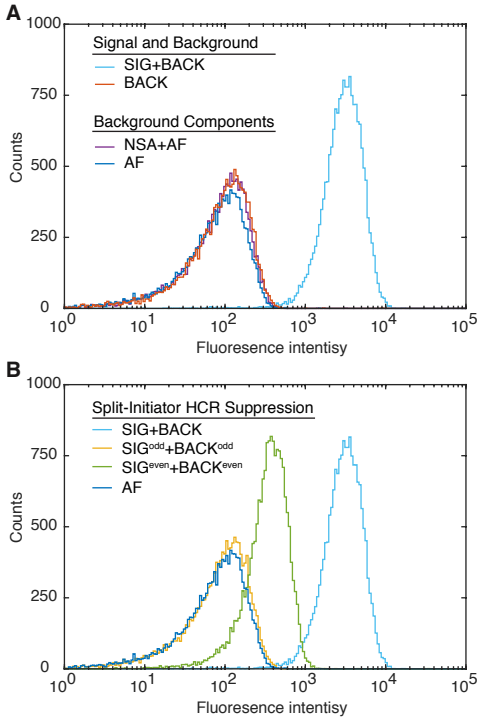

Figure S22. Measurement of signal and background, background components, and split-initiator HCR suppression for *eGFP* transgenic target in *E. coli* (cf. Figure 6A). (A) Signal and background: use experiments of Types 1a and 1b in Table S7A (odd + even probes, hairpins) to measure SIG+BACK (GFP+ cells) and BACK (WT cells). Background components: use experiment of Type 2 in Table S7B (no probes, with hairpins) to measure NSA+AF (GFP+ cells); use experiment of Type 3 (no probes, no hairpins) to measure AF (GFP+ cells). (B) Split-initiator HCR suppression: use experiment of Type 4a in Table S7C (odd probes, hairpins) to measure SIG^{odd}+$BACK^{odd}$ (GFP+ cells); use experiment of Type 5a in Table S7C (even probes, hairpins) to measure SIG^{even}+$BACK^{even}$ (GFP+ cells). Distribution of single-cell fluorescence intensities. Protocol: in situ HCR v3.0 (Section S2.4). Probe set: 12 split-initiator probe pairs. Amplifier: B3-Alexa594. Sample: 18,000 *E. coli* in suspension (WT: K12 MG1655; GFP+: K12 MG1655 pUA66-sdhC expressing *gfpmut2*).

		Channel	Reagents		
	Quantity	B3-Alexa594	Probes	Hairpins	Cell type
A	SIG+NSD+NSA+AF = SIG+BACK	3330 ± 10	odd + even	✓	GFP+
	NSD+NSA+AF = BACK	120 ± 20	odd + even	✓	WT
	SIG	3200 ± 30			
	SIG/BACK	26 ± 5			
B	NSA+AF	72.5 ± 0.7		✓	GFP+
	AF	55.7 ± 0.7		✓	GFP+
	NSA	17 ± 1			
	NSD	50 ± 20			
C	SIG^{odd}+$BACK^{odd}$	71.3 ± 0.7	odd	✓	GFP+
	SIG^{even}+$BACK^{even}$	395 ± 15	even	✓	GFP+
	SIG^{odd}	<1			
	SIG^{even}	320 ± 10			
	SIG/SIG^{odd}	>3000			
	SIG/SIG^{even}	9.9 ± 0.5			

Table S19. Estimated signal-to-background, background components, and split-initiator HCR suppression for *eGFP* transgenic target in *E. coli* (cf. Figure 6A). (A) Signal-to-background (SIG/BACK). (B) Background components (AF, NSA, NSD). (C) Split-initiator HCR suppression (SIG/SIG^{odd}, SIG/SIG^{even}). The signal estimates SIG^{odd} and SIG^{even} are calculated using the background approximation $BACK^{odd}$ = $BACK^{even}$ $\approx$ NSA+AF, which leads to lower bounds on SIG/SIG^{odd} and SIG/SIG^{even}. Mean $\pm$ standard error, $N = 18,000$ cells. Analysis based on single-cell intensities of Figure S22 using methods of Section S1.5.2.

S3.6 qHCR flow cytometry: analog mRNA relative quantitation for high-throughput expression profiling of human and bacterial cells (cf. Figure 6)

S3.6.1 Testing for a crowding effect

In order to perform multiplexed quantitative flow cytometry using HCR, it is important that there is not a crowding effect in which amplification polymers tethered to one target molecule affect the signal intensity for a different target molecule. To test for a possible crowding effect, we analyzed two highly-expressed target mRNAs (*GAPDH* and *ACTB*) individually (1-target studies) and also simultaneously (2-target studies) within HEK cells. Figure S23 compares the signal intensity distributions for 1-target and 2-target studies, revealing similar intensity distributions whether targets were detected alone or together, suggesting that there is not a significant crowding effect (either antagonistic or synergistic).

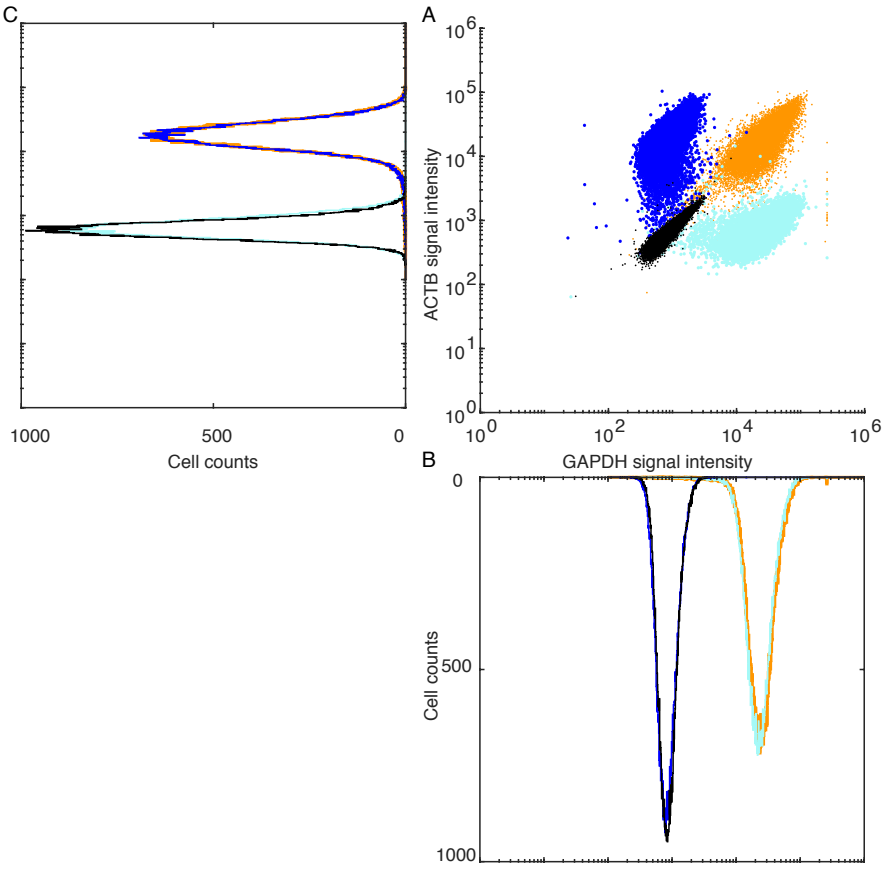

Figure S23. Comparison of signal intensity distributions for individual and multiplexed floHCR of *GAPDH* and *ACTB*. (A) Raw single-cell fluorescence intensity scatter plots: *GAPDH* channel vs *ACTB* channel. (B) Single-cell fluorescence intensity histogram for *GAPDH* channel. (C) Single-cell fluorescence intensity histogram for *ACTB* channel. Orange data: signal plus background for *GAPDH* and *ACTB* . Cyan data: signal plus background for *GAPDH* and autofluoresence for *ACTB* . Blue data: background for *ACTB* and signal plus autofluoresence for *GAPDH*. Black data: autofluoresence for *GAPDH* and *ACTB*. Sample: 65,000 HEK cells in suspension (WT).

S3.6.2 Redundant 2-channel detection of *GAPDH* endogenous target in HEK cells

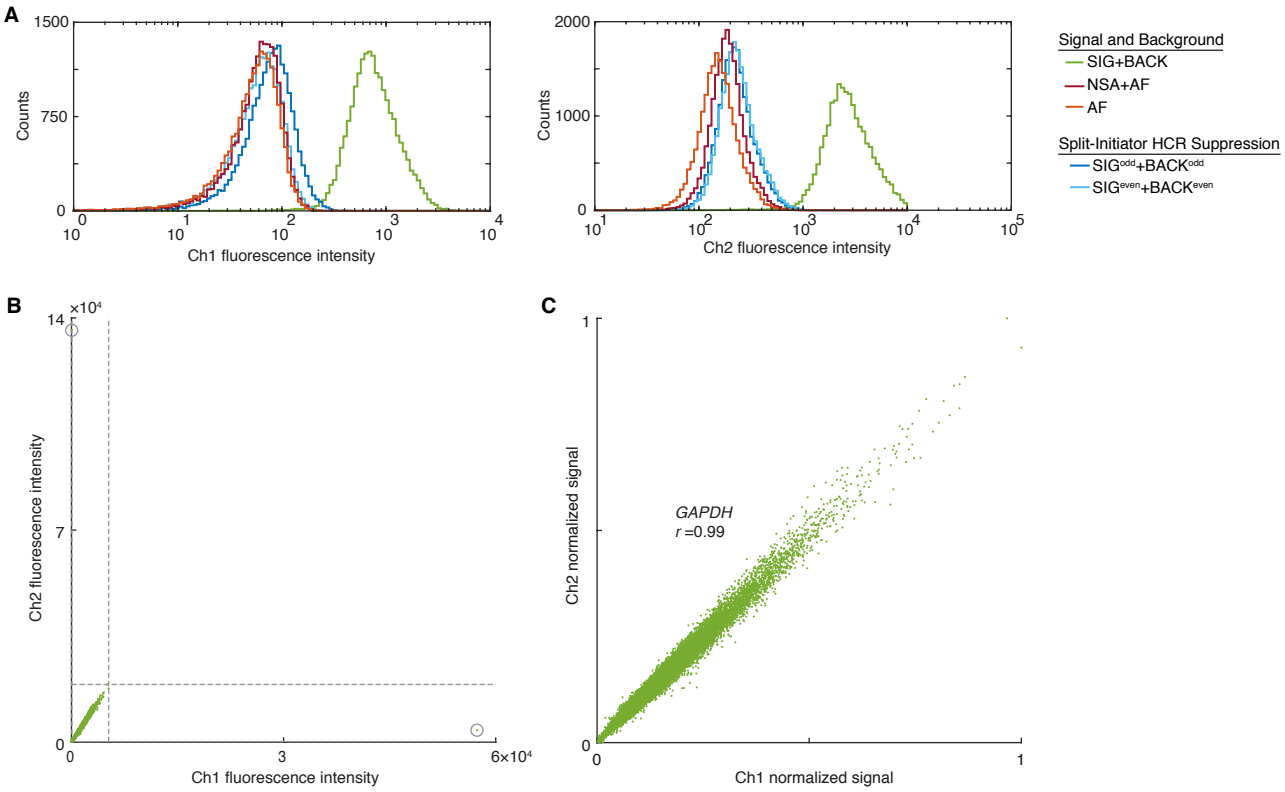

Figure S24. Measurement of signal and background, background components, and split-initiator HCR suppression for redundant 2-channel detection of *GAPDH* endogenous target (cf. Figure 6B). (A) Signal and background: use experiment of Type 1a in Table S8A to measure SIG+BACK (even + odd probes, hairpins). Background components: use experiment of Type 2 in Table S8B (no probes, with hairpins) to measure NSA+AF; use experiment of Type 3 (no probes, no hairpins) to measure AF. Split-initiator HCR suppression: use experiment of Type 4a in Table S8C (odd probes, hairpins) to measure $\mathrm{SIG^{odd}}$+$\mathrm{BACK^{odd}}$ (odd probes, hairpins); use experiment of Type 5a in Table S8C to measure $\mathrm{SIG^{even}}$+$\mathrm{BACK^{even}}$ (even probes, hairpins). Single-cell fluorescence intensity histograms for Ch1 and Ch2. (B) Raw single-cell fluorescence intensity scatter plots representing signal plus background. Dashed lines represent BOT and TOP values (Table S20) used to normalize data for panel C using methods of of Section S1.5.4 (outliers excluded from normalized scatter plots marked with circles). (C) Normalized single-cell fluorescence intensity scatter plots representing estimated normalized signal (Pearson correlation coefficient, r). Protocol: in situ HCR v3.0 (Section S2.3). Probe sets: 10 split-initiator probe pairs per channel. Amplifiers: B5-Alexa488 (Ch1) and B4-Alexa594 (Ch2). Sample: 20,000 HEK cells in suspension (WT).

		Channel		Reagents		
	Quantity	Ch1: B5-Alexa488	Ch2: B4-Alexa594	Probes	Hairpins	Cell type
A	SIG+BACK	870 ± 5	3105 ± 14	odd + even	✓	WT
	SIG	808 ± 5	2904 ± 14			
	SIG/BACK	12.86 ± 0.08	14.43 ± 0.08			
B	NSA+AF	62.8 ± 0.2	201.2 ± 0.6		✓	WT
	AF	57.4 ± 0.2	166.5 ± 0.5		✓	WT
	NSA	5.4 ± 0.3	34.7 ± 0.8			
C	SIG^{odd}+$BACK^{odd}$	82.8 ± 0.3	244.5 ± 0.8	odd	✓	WT
	SIG^{even}+$BACK^{even}$	63.0 ± 0.2	256.6 ± 0.8	even	✓	WT
	SIG^{odd}	20.0 ± 0.4	43 ± 1			
	SIG^{even}	<0.3	55 ± 1			
	SIG/SIG^{odd}	40.4 ± 0.8	67 ± 2			
	SIG/SIG^{even}	>3000	52 ± 1			
D	BOT	62.8	201.2			
	TOP	5265.2	19 049.4			

Table S20. Estimated signal-to-background, background components, and split-initiator HCR suppression for redundant 2-channel detection of *GAPDH* endogenous target (cf. Figure 6B). (A) Signal-to-background (SIG/BACK). (B) Background components (AF, NSA). (C) Split-initiator HCR suppression (SIG/SIG^{odd}, SIG/SIG^{even}). The signal estimates SIG, SIG^{odd} and SIG^{even} are calculated using the background approximation BACK = $BACK^{odd}$ = $BACK^{even}$ ≈ NSA+AF. Mean ± standard error, $N = 20,000$ cells. Analysis based on single-cell intensities of Figure S24 using methods of Section S1.5.3. (D) BOT and TOP values used to calculate normalized single-cell intensities for scatter plots of Figures 6B and S24C using methods of Section S1.5.4.

S3.6.3 Redundant 2-channel detection of *PGK1* endogenous target in HEK cells

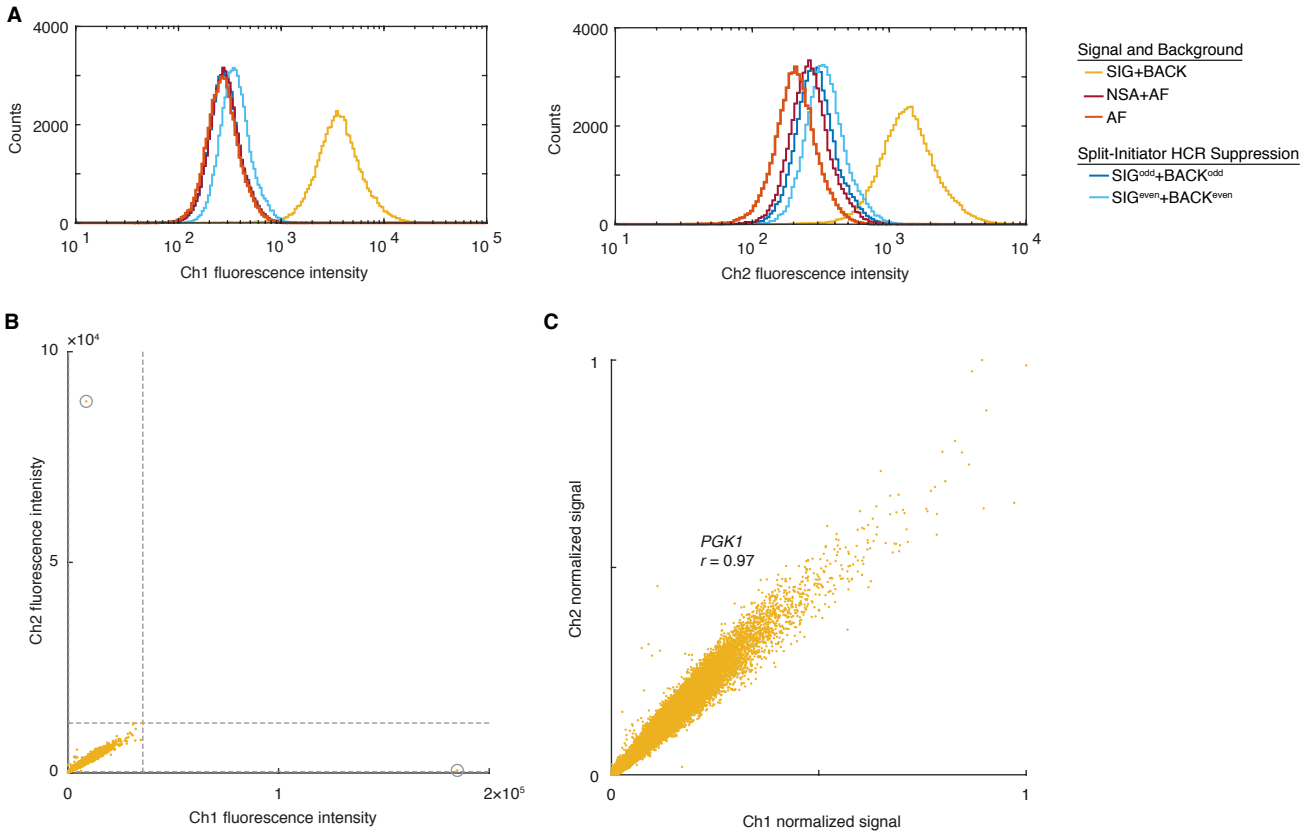

Figure S25. Measurement of signal and background, background components, and split-initiator HCR suppression for redundant 2-channel detection of *PGK1* endogenous target. (A) Signal and background: use experiment of Type 1a in Table S8A to measure SIG+BACK (even + odd probes, hairpins). Background components: use experiment of Type 2 in Table S8B (no probes, with hairpins) to measure NSA+AF; use experiment of Type 3 (no probes, no hairpins) to measure AF. Split-initiator HCR suppression: use experiment of Type 4a in Table S8C (odd probes, hairpins) to measure SIG^{odd}+$BACK^{odd}$ (odd probes, hairpins); use experiment of Type 5a in Table S8C to measure SIG^{even}+$BACK^{even}$ (even probes, hairpins). Single-cell fluorescence intensity histograms for Ch1 and Ch2. (B) Raw single-cell fluorescence intensity scatter plots representing signal plus background. Dashed lines represent BOT and TOP values (Table S21) used to normalize data for panel C using methods of of Section S1.5.4 (outliers excluded from normalized scatter plots marked with circles). (C) Normalized single-cell fluorescence intensity scatter plots representing estimated normalized signal (Pearson correlation coefficient, r). Protocol: in situ HCR v3.0 (Section S2.3). Probe sets: 18 split-initiator probe pairs per channel. Amplifiers: B1-Alexa488 (Ch1) and B2-Alexa594 (Ch2). Sample: 54,000 HEK cells in suspension (WT).

		Channel		Reagents		
	Quantity	Ch1: B1-Alexa488	Ch2: B2-Alexa594	Probes	Hairpins	Cell type
A	SIG+BACK	4145 ± 11	1528 ± 4	odd + even	✓	WT
	SIG	3843 ± 11	1248 ± 4			
	SIG/BACK	12.72 ± 0.04	4.47 ± 0.02			
B	NSA+AF	302.1 ± 0.5	279.4 ± 0.5		✓	WT
	AF	289.3 ± 0.5	220.1 ± 0.4		✓	WT
	NSA	12.9 ± 0.7	59.3 ± 0.6			
C	SIG^{odd}+$BACK^{odd}$	301.3 ± 0.5	309.5 ± 0.6	odd	✓	WT
	SIG^{even}+$BACK^{even}$	380.5 ± 0.6	374 ± 7	even	✓	WT
	SIG^{odd}	< 0.7	30.1 ± 0.8			
	SIG^{even}	78.4 ± 0.8	94 ± 7			
	SIG/SIG^{odd}	>5000	41 ± 1			
	SIG/SIG^{even}	49.0 ± 0.5	13 ± 1			
D	BOT	302.1	279.4			
	TOP	35 538.8	11 847.7			

Table S21. Estimated signal-to-background, background components, and split-initiator HCR suppression for redundant 2-channel detection of *PGK1* endogenous target. (A) Signal-to-background (SIG/BACK). (B) Background components (AF, NSA). (C) Split-initiator HCR suppression (SIG/SIG^{odd}, SIG/SIG^{even}). The signal estimates SIG, SIG^{odd} and SIG^{even} are calculated using the background approximation BACK = $BACK^{odd}$ = $BACK^{even}$ $\approx$ NSA+AF. Mean $\pm$ standard error, $N = 54,000$ cells. Analysis based on single-cell intensities of Figure S25 using methods of Section S1.5.3. (D) BOT and TOP values used to calculate normalized single-cell intensities for scatter plots of Figure S25C using methods of Section S1.5.4.

S3.6.4 Redundant 2-channel detection of *fusA* endogenous target in *E. coli*

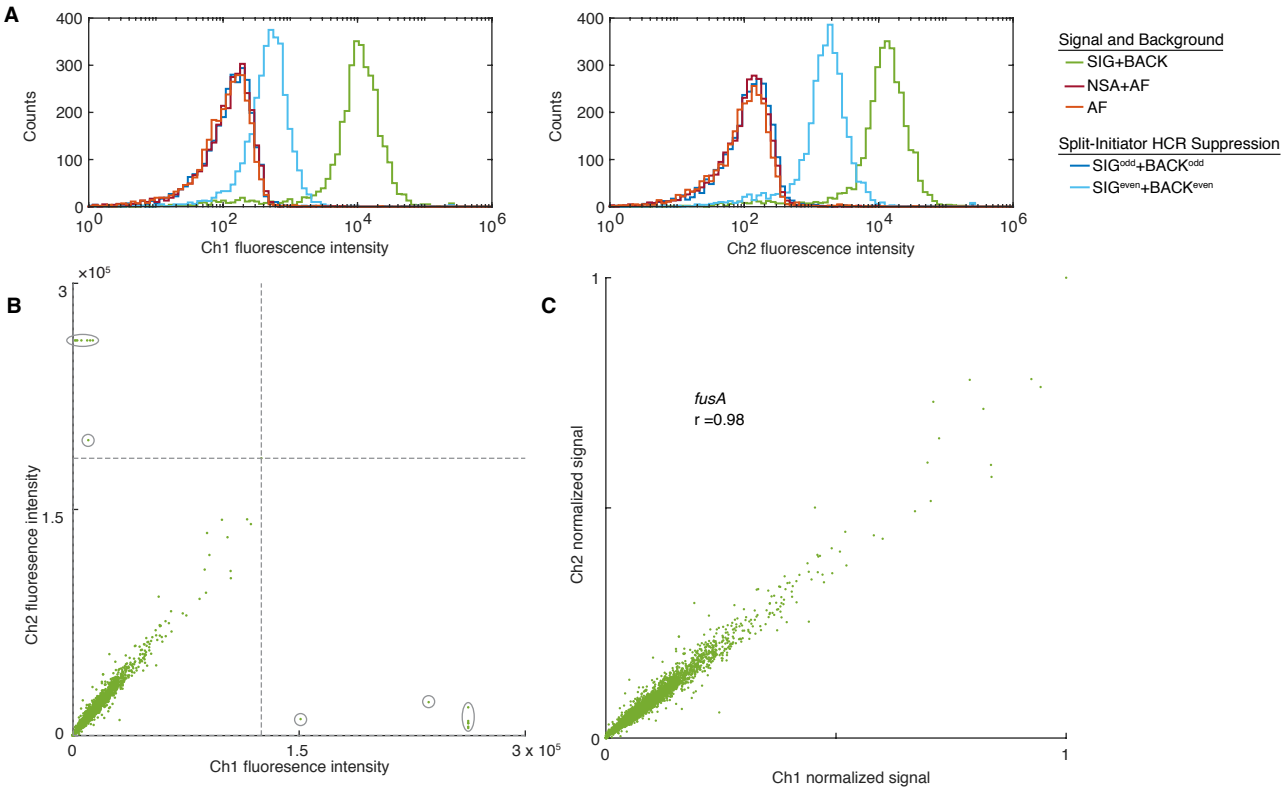

Figure S26. Measurement of signal and background, background components, and split-initiator HCR suppression for redundant 2-channel detection of *fusA* endogenous target (cf. Figure 6B). (A) Signal and background: use experiment of Type 1a in Table S8A to measure SIG+BACK (even + odd probes, hairpins). Background components: use experiment of Type 2 in Table S8B (no probes, with hairpins) to measure NSA+AF; use experiment of Type 3 (no probes, no hairpins) to measure AF. Split-initiator HCR suppression: use experiment of Type 4a in Table S8C (odd probes, hairpins) to measure $\mathrm{SIG^{odd}}$+$\mathrm{BACK^{odd}}$ (odd probes, hairpins); use experiment of Type 5a in Table S8C to measure $\mathrm{SIG^{even}}$+$\mathrm{BACK^{even}}$ (even probes, hairpins). Single-cell fluorescence intensity histograms for Ch1 and Ch2. (B) Raw single-cell fluorescence intensity scatter plots representing signal plus background. Dashed lines represent BOT and TOP values (Table S22) used to normalize data for panel C using methods of of Section S1.5.4 (outliers excluded from normalized scatter plots marked with ellipses). (C) Normalized single-cell fluorescence intensity scatter plots representing estimated normalized signal (Pearson correlation coefficient, r). Protocol: in situ HCR v3.0 (Section S2.4). Probe sets: 18 split-initiator probe pairs per channel. Amplifiers: B3-Alexa488 (Ch1) and B2-Alexa594 (Ch2). Sample: 3,400 *E. coli* K12 MG1655 in suspension (WT).

		Channel		Reagents		
	Quantity	Ch1: B3-Alexa488	Ch2: B2-Alexa594	Probes	Hairpins	Cell type
A	SIG+BACK	13 100 ± 300	15 800 ± 300	odd + even	✓	WT
	SIG	13 000 ± 300	15 700 ± 300			
	SIG/BACK	99 ± 3	135 ± 7			
B	NSA+AF	130 ± 3	116 ± 5		✓	WT
	AF	126 ± 7	120 ± 10		✓	WT
	NSA	< 7	< 14			
C	SIG^{odd}+$BACK^{odd}$	500 ± 100	400 ± 100	odd	✓	WT
	SIG^{even}+$BACK^{even}$	1100 ± 200	2600 ± 200	even	✓	WT
	SIG^{odd}	300 ± 100	300 ± 100			
	SIG^{even}	900 ± 200	2500 ± 200			
	SIG/SIG^{odd}	40 ± 20	50 ± 20			
	SIG/SIG^{even}	14 ± 3	6.2 ± 0.6			
D	BOT	130.3	116			
	TOP	150 951.6	183 947.3			

Table S22. Estimated signal-to-background, background components, and split-initiator HCR suppression for redundant 2-channel detection of *fusA* endogenous target (cf. Figure 6B). (A) Signal-to-background (SIG/BACK). (B) Background components (AF, NSA). (C) Split-initiator HCR suppression (SIG/SIG^{odd}, SIG/SIG^{even}). The signal estimates SIG, SIG^{odd} and SIG^{even} are calculated using the background approximation BACK = $BACK^{odd}$ = $BACK^{even}$ ≈ NSA+AF. Mean ± standard error, $N = 3,400$ cells. Analysis based on single-cell intensities of Figure S26 using methods of Section S1.5.3. (D) BOT and TOP values used to calculate normalized single-cell intensities for scatter plots of Figures 6B and S26C using methods of Section S1.5.4.

S3.6.5 Multiplexed 2-channel detection of *GAPDH* and *PGK1* endogenous targets in HEK cells

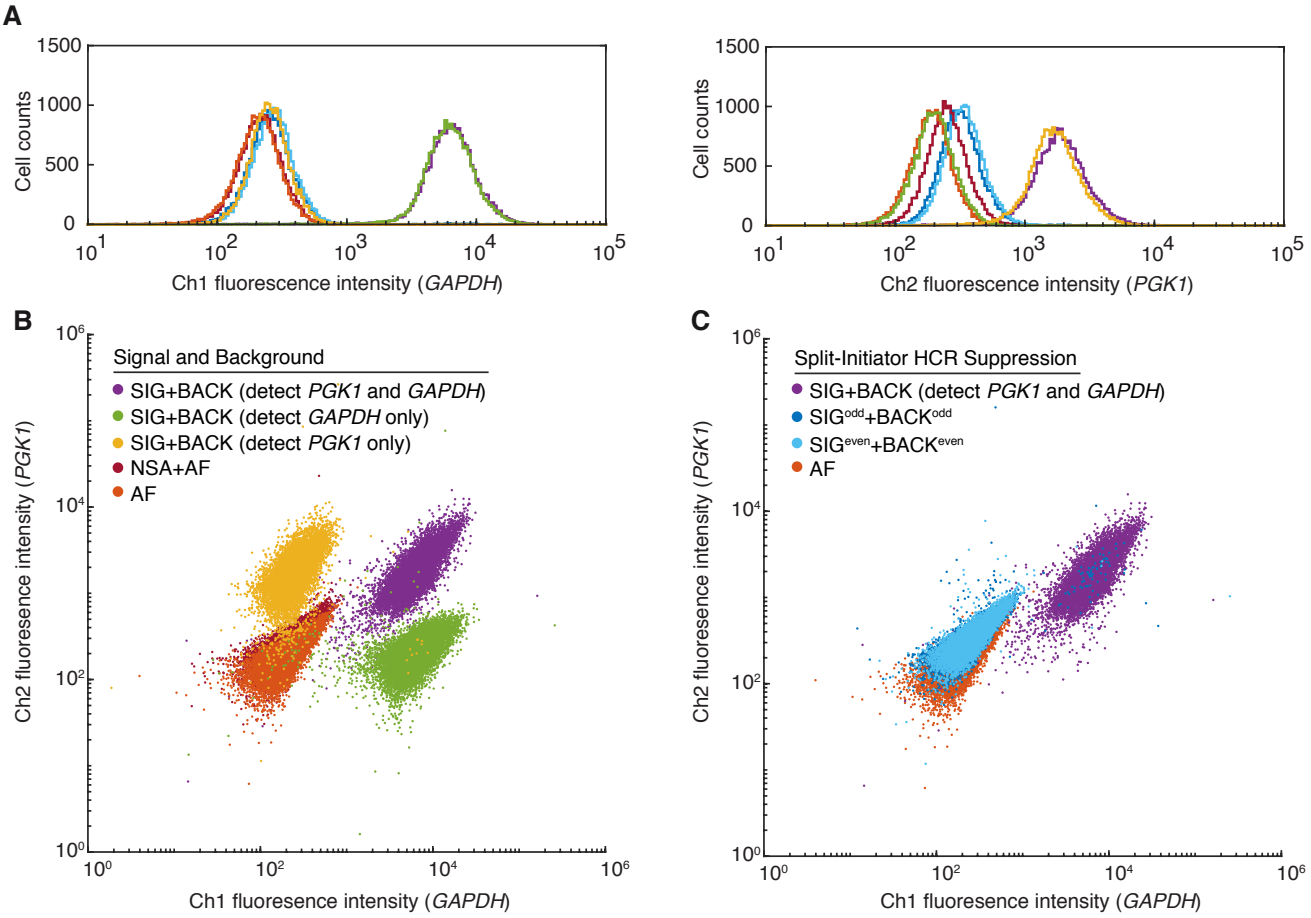

Figure S27. Measurement of signal and background, background components, and split-initiator HCR suppression for multiplexed 2-channel detection of *GAPDH* and *PGK1* endogenous targets. (A) Single-cell fluorescence intensity histograms for Ch1 and Ch2. (B) Raw single-cell fluorescence intensity scatter plots for Ch1 vs Ch2. Signal and background: use experiment of Type 1a in Table S8A to measure SIG+BACK (even + odd probes, hairpins). Background components: use experiment of Type 2 in Table S8B (no probes, with hairpins) to measure NSA+AF; use experiment of Type 3 (no probes, no hairpins) to measure AF. (C) Split-initiator HCR suppression: use experiment of Type 4a in Table S8C (odd probes, hairpins) to measure SIG^{odd}+$BACK^{odd}$ (odd probes, hairpins); use experiment of Type 5a in Table S8C to measure SIG^{even}+$BACK^{even}$ (even probes, hairpins). Protocol: in situ HCR v3.0 (Section S2.3). Ch1: target mRNA *GAPDH*, probe set with 10 split-initiator probe pairs, amplifier B4-Alexa488. Ch2: target mRNA *PGK1*, probe set with 18 split-initiator probe pairs, amplifier B2-Alexa594. Sample: 18,000 HEK cells in suspension (WT).

		Channel		Reagents		
	Quantity	Ch1: B4-Alexa488	Ch2: B2-Alexa594	Probes	Hairpins	Cell type
A	SIG+BACK	6980 $\pm$ 30	2073 $\pm$ 8	odd + even	✓	WT
	SIG	6760 $\pm$ 30	1806 $\pm$ 8			
	SIG/BACK	29.6 $\pm$ 0.1	6.77 $\pm$ 0.05			
B	NSA+AF	228.2 $\pm$ 0.7	266.6 $\pm$ 1.5		✓	WT
	AF	219.7 $\pm$ 0.6	198.4 $\pm$ 0.6		✓	WT
	NSA	8.4 $\pm$ 0.9	68 $\pm$ 2			
C	SIG^{odd}+$BACK^{odd}$	301 $\pm$ 5	355 $\pm$ 9	odd	✓	WT
	SIG^{even}+$BACK^{even}$	303 $\pm$ 14	362.8 $\pm$ 1.2	even	✓	WT
	SIG^{odd}	73 $\pm$ 5	88 $\pm$ 9			
	SIG^{even}	74 $\pm$ 14	96 $\pm$ 2			
	SIG/SIG^{odd}	93 $\pm$ 6	21 $\pm$ 2			
	SIG/SIG^{even}	91 $\pm$ 14	18.8 $\pm$ 0.4			

Table S23. Estimated signal-to-background, background components, and split-initiator HCR suppression for multiplexed 2-channel detection of *GAPDH* and *PGK1* endogenous targets. (A) Signal-to-background (SIG/BACK). (B) Background components (AF, NSA). (C) Split-initiator HCR suppression (SIG/SIG^{odd}, SIG/SIG^{even}). The signal estimates SIG, SIG^{odd} and SIG^{even} are calculated using the background approximation BACK = $BACK^{odd}$ = $BACK^{even}$ $\approx$ NSA+AF. Mean $\pm$ standard error, $N = 18,000$ cells. Analysis based on single-cell intensities of Figure S27 using methods of Section S1.5.3.

S3.6.6 Multiplexed 2-channel detection of *fusA* and *icd* endogenous targets in *E. coli*

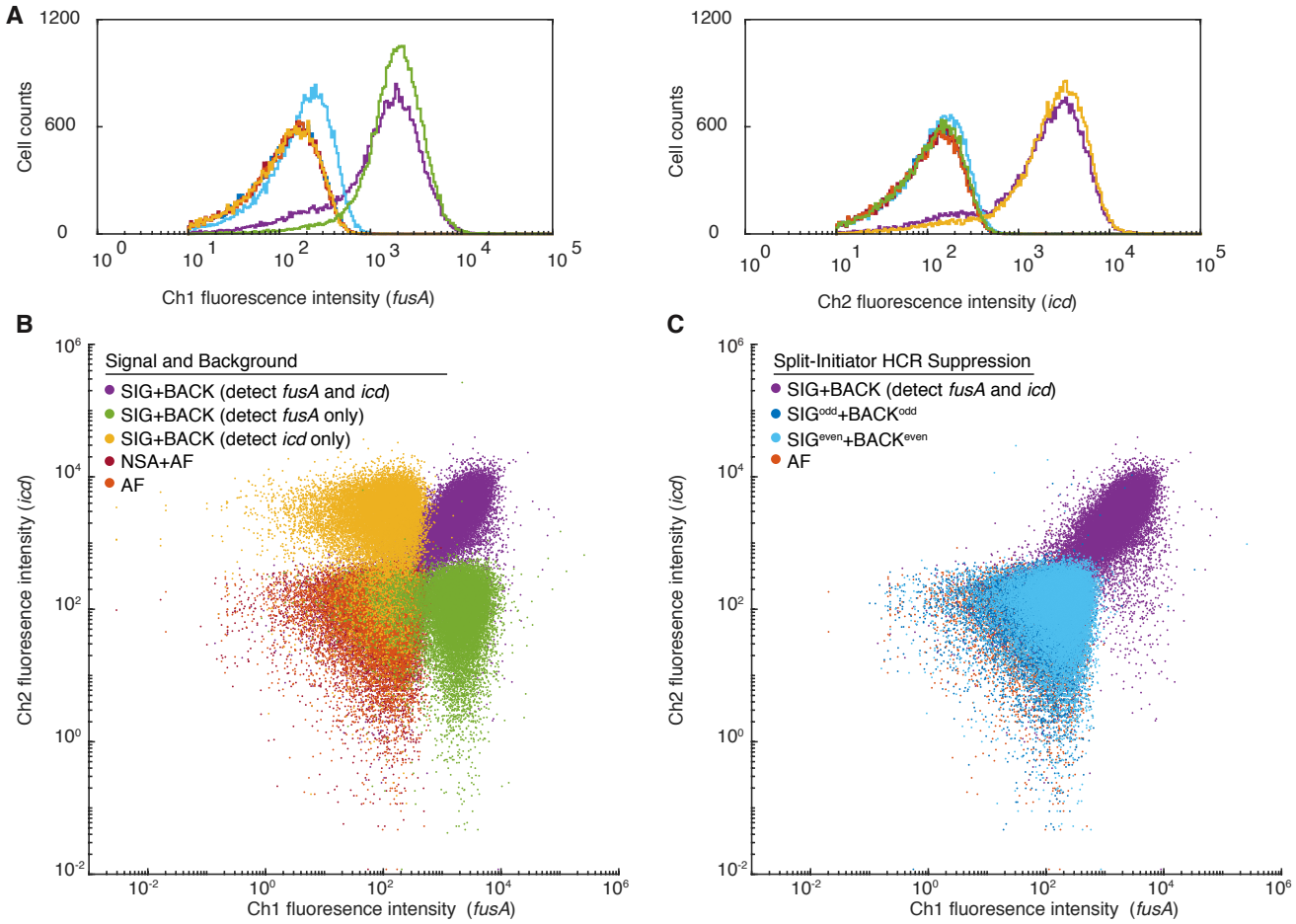

Figure S28. Measurement of signal and background, background components, and split-initiator HCR suppression for multiplexed 2-channel detection of *fusA* and *icd* endogenous targets. (A) Single-cell fluorescence intensity histograms for Ch1 and Ch2. (B) Raw single-cell fluorescence intensity scatter plots for Ch1 vs Ch2. Signal and background: use experiment of Type 1a in Table S8A to measure SIG+BACK (even + odd probes, hairpins). Background components: use experiment of Type 2 in Table S8B (no probes, with hairpins) to measure NSA+AF; use experiment of Type 3 (no probes, no hairpins) to measure AF. (C) Split-initiator HCR suppression: use experiment of Type 4a in Table S8C (odd probes, hairpins) to measure SIG^{odd}+$BACK^{odd}$ (odd probes, hairpins); use experiment of Type 5a in Table S8C to measure SIG^{even}+$BACK^{even}$ (even probes, hairpins). Protocol: in situ HCR v3.0 (Section S2.4). Ch1: target mRNA *fusA*, probe set with 18 split-initiator probe pairs, amplifier B3-Alexa488. Ch2: target mRNA *icd*, probe set with 20 split-initiator probe pairs, amplifier B1-Alexa594. Sample: 35,000 *E. coli* K12 MG1655 in suspension (WT).

		Channel		Reagents		
	Quantity	Ch1: B3-Alexa488	Ch2: B1-Alexa594	Probes	Hairpins	Cell type
A	SIG+BACK	1756 ± 9	2533 ± 12	odd + even	✓	WT
	SIG	1673 ± 9	2470 ± 12			
	SIG/BACK	20.1 ± 0.2	38.9 ± 0.5			
B	NSA+AF	83.2 ± 0.7	63.5 ± 0.7		✓	WT
	AF	82.3 ± 0.7	60.1 ± 0.7		✓	WT
	NSA	1 ± 1	3.4 ± 0.9			
C	SIG^{odd}+$BACK^{odd}$	86 ± 1	66.6 ± 0.8	odd	✓	WT
	SIG^{even}+$BACK^{even}$	180 ± 8	93 ± 1	even	✓	WT
	SIG^{odd}	3 ± 1	3 ± 1			
	SIG^{even}	97 ± 8	29 ± 1			
	SIG/SIG^{odd}	600 ± 300	800 ± 300			
	SIG/SIG^{even}	17.2 ± 1.3	85 ± 4			

Table S24. Estimated signal-to-background, background components, and split-initiator HCR suppression for multiplexed 2-channel detection of *fusA* and *icd* endogenous targets. (A) Signal-to-background (SIG/BACK). (B) Background components (AF, NSA). (C) Split-initiator HCR suppression (SIG/SIG^{odd}, SIG/SIG^{even}). The signal estimates SIG, SIG^{odd} and SIG^{even} are calculated using the background approximation BACK = $BACK^{odd}$ = $BACK^{even}$ $\approx$ NSA+AF. Mean $\pm$ standard error, $N = 35,000$ cells. Analysis based on single-cell intensities of Figure S28 using methods of Section S1.5.3.

S3.7 dHCR imaging: digital mRNA absolute quantitation in an anatomical context (cf. Figure 7)

S3.7.1 Redundant 2-channel detection of single *BRAF* mRNAs in HEK cells using in situ HCR v3.0

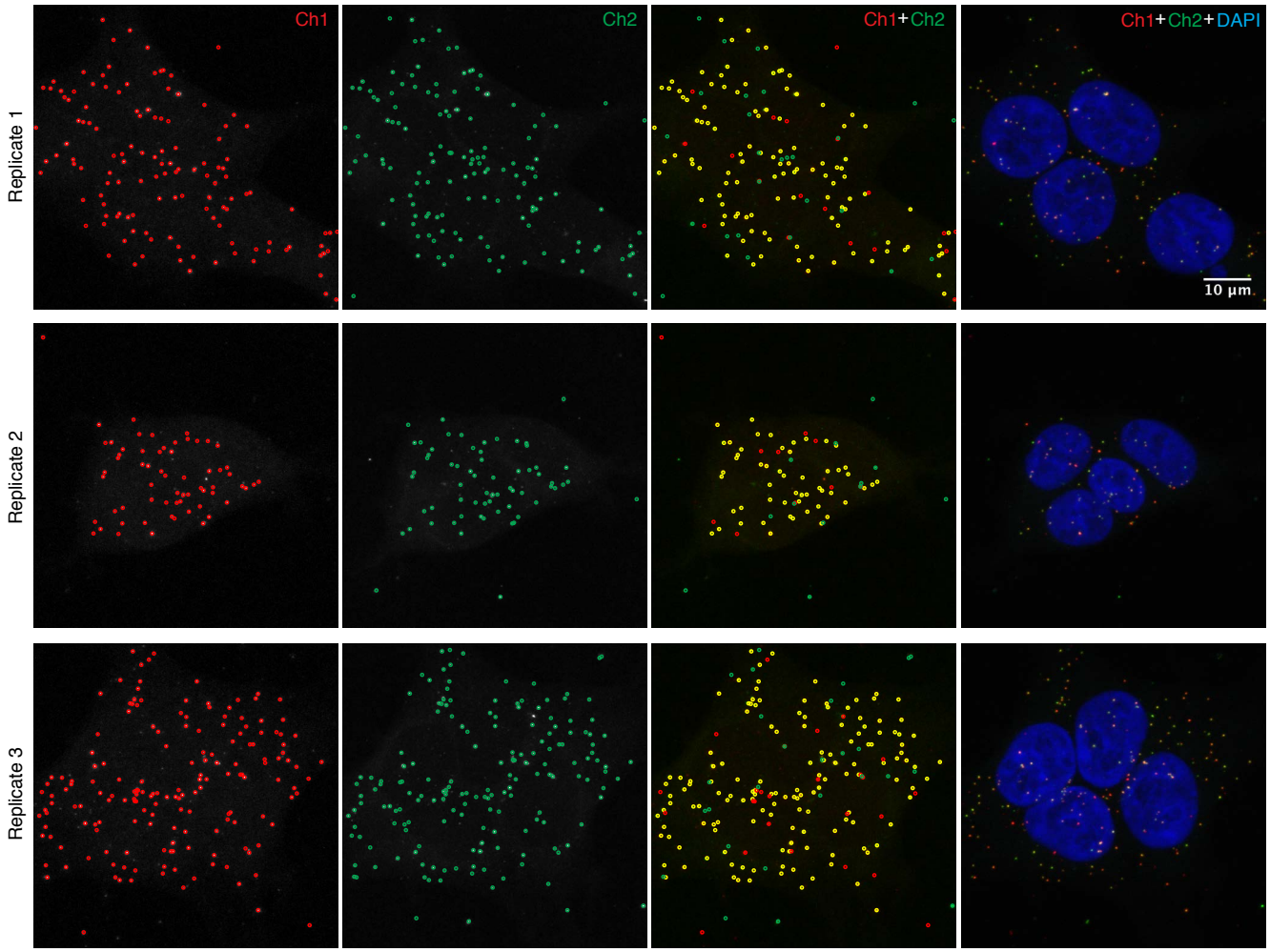

Figure S29. Redundant 2-channel detection of single *BRAF* mRNAs in HEK cells using in situ HCR v3.0 (cf. Figure 7A). Confocal images: individual channels and merge (without and with DAPI nuclear stain). Maximum intensity projection in the axial direction over 7.14 μm (17 focal planes). Pixel size: 0.062×0.062 μm. Probe sets: 23 split-initiator probe pairs per channel. Amplifiers: B3-Alexa647 (Ch1) and B4-Alexa546 (Ch2). Red circles: dots detected in Ch1. Green circles: dots detected in Ch2. Yellow circles: dots detected in both channels.

	Dots		Colocalized dots	Colocalization fractions	
	N_1	N_2	N_{12}	C_1	C_2
Replicate 1	129	136	110	0.85	0.81
Replicate 2	63	65	53	0.84	0.82
Replicate 3	170	170	144	0.85	0.85
Mean				0.85 ± 0.003	0.82 ± 0.01

Table S25. Dot colocalization fractions for redundant 2-channel detection of single *BRAF* mRNAs in HEK cells using in situ HCR v3.0 (cf. Figure 7A). Mean ± standard error, $N = 3$ replicate samples. Analysis based on the images of Figure S29 using the methods of Section S1.4.6 with the settings in Table S6.

S3.7.2 Redundant 2-channel detection of single *Dmbx1* mRNAs in whole-mount chicken embryos using in situ HCR v3.0

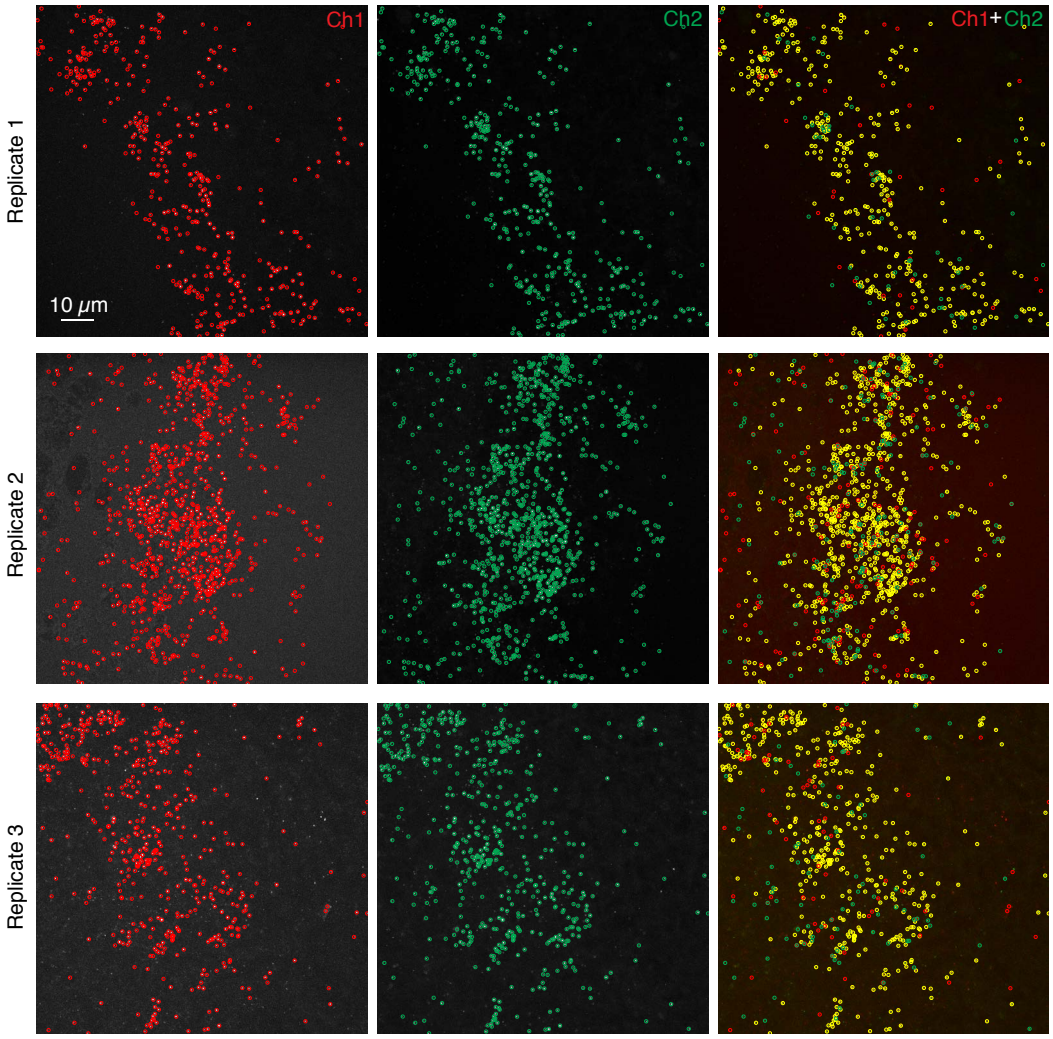

Figure S30. Redundant 2-channel detection of single *Dmbx1* mRNAs in whole-mount chicken embryos using in situ HCR v3.0 (cf. Figure 7B). Confocal images: individual channels and merge. Maximum intensity projection in the axial direction over 5.04-23.52 μm (12, 54, 56 focal planes for replicates 1, 2, 3 depending on sample thickness). Pixel size: 0.099×0.099 μm. Probe sets: 25 split-initiator probe pairs per channel. Amplifiers: B2-Alexa647 (Ch1) and B1-Alexa594 (Ch2). Embryos fixed stage HH 8. Red circles: dots detected in Ch1. Green circles: dots detected in Ch2. Yellow circles: dots detected in both channels.

	Dots		Colocalized dots	Colocalization fractions	
	N_1	N_2	N_{12}	C_1	C_2
Replicate 1	403	417	364	0.90	0.87
Replicate 2	992	990	794	0.80	0.80
Replicate 3	526	539	448	0.85	0.83
Mean				0.85 ± 0.03	0.84 ± 0.02

Table S26. Dot colocalization fractions for redundant 2-channel detection of single *Dmbx1* mRNAs in whole-mount chicken embryos using in situ HCR v3.0 (cf. Figure 7B). Mean ± standard error, $N = 3$ replicate embryos. Analysis based on the images of Figure S30 using the methods of Section S1.4.6 with the settings in Table S6.

S3.7.3 Redundant 2-channel detection of single *kdrl* mRNAs in whole-mount zebrafish embryos using in situ HCR v2.0 (Shah *et al.*, 2016)

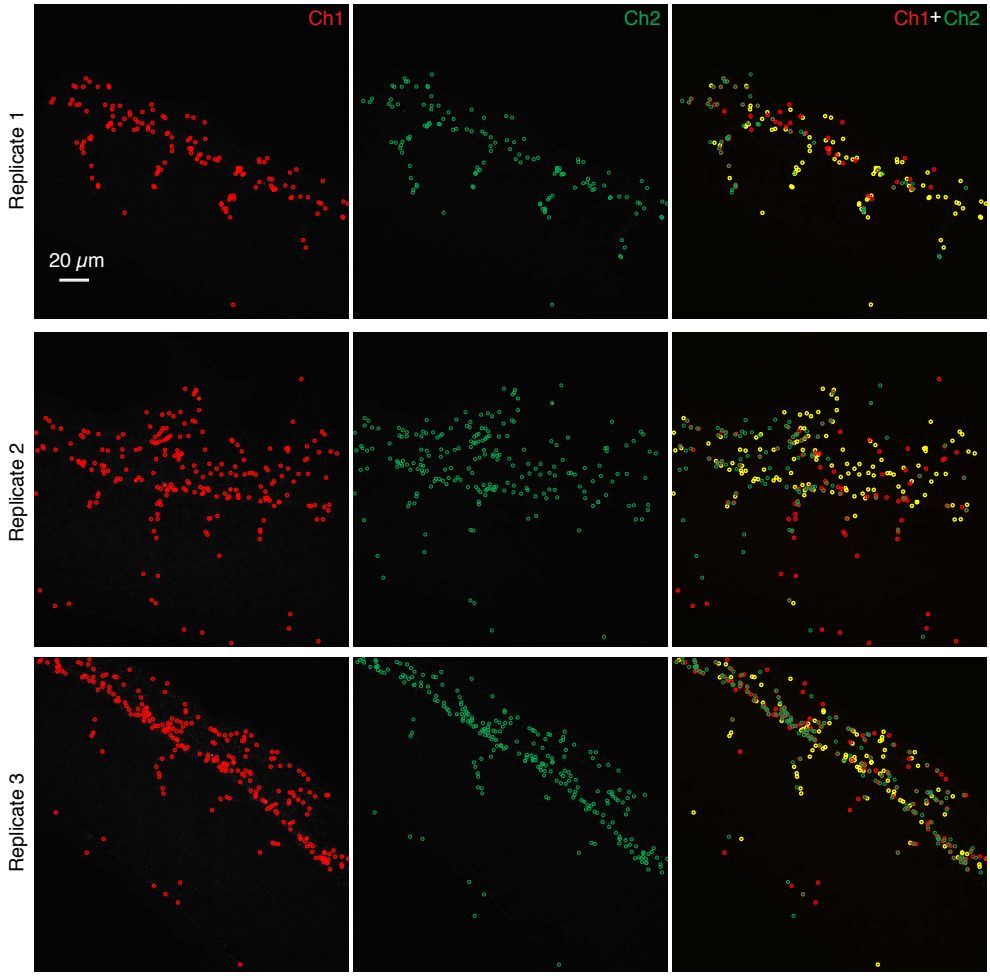

Figure S31. Redundant 2-channel detection of single *kdrl* mRNAs in whole-mount zebrafish embryos using in situ HCR v2.0. Spinning disk confocal images: individual channels and merge from Shah *et al.* (2016). Maximum intensity projection in the axial direction over 13 μm (39 focal planes). Pixel size: 0.217×0.217 μm. Probe sets: 39 standard probes per channel, each incorporating a 30-nt target-binding domain and a full HCR initiator. Amplifiers: B3-Alexa647 (Ch1) and B2-Alexa546 (Ch2). Embryos fixed 27 hpf. Red circles: dots detected in Ch1. Green circles: dots detected in Ch2. Yellow circles: dots detected in both channels.

	Dots		Colocalized dots	Colocalization fractions	
	N_1	N_2	N_{12}	C_1	C_2
Replicate 1	139	132	79	0.57	0.60
Replicate 2	220	215	113	0.51	0.53
Replicate 3	243	245	91	0.37	0.37
Mean				0.49 ± 0.06	0.50 ± 0.07

Table S27. Dot colocalization fractions for redundant 2-channel detection of single *kdrl* mRNAs in whole-mount zebrafish embryos using in situ HCR v3.0. Mean ± standard error, $N = 3$ replicate embryos. Analysis based on the images of Figure S31 using the methods of Section S1.4.6 with the settings in Table S6.

S4 Probe sequences

Target mRNA sequences were obtained from the National Center for Biotechnology Information (NCBI) (McEntyre & Ostell, 2002) with the exception of *d2eGFP* (pd2EGFP-1, Clontech, Cat. #6008-1). Spatial and temporal expression information for whole-mount chicken embryos were obtained from the Gallus Expression in Situ Hybridization Analysis (GEISHA) (Bell *et al.*, 2004; Darnell *et al.*, 2007). Within a given probe set, each DNA standard probe or split-initiator probe pair initiates the same DNA HCR amplifier. Sequences are listed $5'$ to $3'$. Probes are numbered consecutively moving along a target mRNA. For redundant detection experiments, two probe sets are used with each probe set taking alternating probe pairs from along the target (this leads to non-consecutive numbers within each probe set).

S4.1 Standard probes for Figures 3, S5, and S7

Organism: ***G. gallus domesticus***
Target mRNA: **SRY (sex determining region Y)-box 10 (*Sox10*)**
Probe set: **5, 10, or 20 probes (each carrying 2 HCR initiators)**
HCR amplifier: **B3-Alexa647**

#	Initiator I1	Spacer	Probe Sequence (50 nt)	Spacer	Initiator I2
1	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	CATggACCCgTCACTCCATgTCTTgAgTCTTCCTCATCTAgAAggCCAAT	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
2	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	CCAgCAgggATCAAgATTCATgCATgTgTgAATCTTAggCAggACTgCTg	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
3	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	CgggCTATgAAATgAgAAAggCTAAggCTgACAgTgCAgTTCCTgAATCC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
4	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	TTCACgTTTTCAgCAgACACAgTCAAATgCTggAggAgCAAggACCTggT	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
5	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	ATTggAACCACATCTgggTgTTggCAAgTgCATggTAgCTTTCTTggTgC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
6	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	AgAggCggggAgAAAAgCTATAgCgTgCAgCTgTgAAAATCAgCAAggAA	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
7	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	ATAAAATCCATgCAggAAggggTgTgggATTAAACAgATgggACAgggggg	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
8	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	gATggCgATAATgTgATgAACAAACgAgCAgTgATgTACACCCCATCggC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
9	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	ATCCACgAgAgTATCTTTCCATCCTgAgTgAAAgTAggAgggAggTgCTg	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
10	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	ACCCgTTAgAAggTCCCACAACACATCTCTCTgATCAgTTgTCAggAgTC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
11	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	CCggCgAgAggCAgTggTggTCTTCAgAACCCACTgggCTCATCTCCACC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
12	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	CCTCgCCCTgCgTggCCTTgCCATTTTTCCgCCTCCgTggCTggTACTTg	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
13	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	TTCTTgTAgTgAgCCTggATAgAggCAgCCCCgCCAgCCTCCCCCTCCAC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
14	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	TgCCCATCggACATgggTgACCCTTCCCCAggATgCCTgTggTCCAggTg	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
15	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	TTAggggTggTgggAggAgTgggAgggCCgTggCTCTgACCTgAAgAgTg	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
16	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	CCCCAAgggAACgCCCTTCTCgCTTggAgTCAgCTTTgCCTgCCTgCAgC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
17	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	ggCCTgggTggCCggCgTgTCCgTTgggTggCAggTATTggTCAAATTCg	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
18	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	TCCATgCTgCTTggAgATCCAggCTgAgTgTCCACTggCCgCAgCCAggg	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
19	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	AACCCTgTgAAggCTgCAgCTCCTgTCCCAgTgCCCCCCTgTTCTCCCTC	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC
20	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	gggTCCAgTCATAgCCgCTCAgCACCTggCTgACCgCTTCACggATgCAg	TAAAA	AAAgTCTAATCCgTCCCTgCCTCTATATCTCCACTC

S4.2 Split-initiator probes for Figures 3, S6, S8, S9, and S10

Organism: ***G. gallus domesticus***
Target mRNA: **SRY (sex determining region Y)-box 10 (*Sox10*)**
Probe set: **5, 10, or 20 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B3-Alexa647**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
1	gTCCCTgCCTCTATATCT	TT	gTCTTCCTCATCTAgAAggCCAATA
3	gTCCCTgCCTCTATATCT	TT	TgTgAATCTTAggCAggACTgCTgC
5	gTCCCTgCCTCTATATCT	TT	gCTgACAgTgCAgTTCCTgAATCCT
7	gTCCCTgCCTCTATATCT	TT	TgCTggAggAgCAAggACCTggTCT
9	gTCCCTgCCTCTATATCT	TT	CAAgTgCATggTAgCTTTCTTggTg
11	gTCCCTgCCTCTATATCT	TT	AgCTgTgAAAATCAgCAAggAAgCA
13	gTCCCTgCCTCTATATCT	TT	gggATTAAACAgATgggACAggggg
15	gTCCCTgCCTCTATATCT	TT	AgCAgTgATgTACACCCCATCggCC
17	gTCCCTgCCTCTATATCT	TT	TgAgTgAAAgTAggAgggAggTgCT
19	gTCCCTgCCTCTATATCT	TT	CTCTCTgATCAgTTgTCAggAgTCA
21	gTCCCTgCCTCTATATCT	TT	gAACCCACTgggCTCATCTCCACCT
23	gTCCCTgCCTCTATATCT	TT	TTCCgCCTCCgTggCTggTACTTgT
25	gTCCCTgCCTCTATATCT	TT	AgCCCCgCCAgCCTCCCCCTCCACC
27	gTCCCTgCCTCTATATCT	TT	CCCAggATgCCTgTggTCCAggTgg
29	gTCCCTgCCTCTATATCT	TT	gCCgTggCTCTgACCTgAAgAgTgC
31	gTCCCTgCCTCTATATCT	TT	gAgTCAgCTTTgCCTgCCTgCAgCT
33	gTCCCTgCCTCTATATCT	TT	ggTggCAggTATTggTCAAATTCgT
35	gTCCCTgCCTCTATATCT	TT	AgTgTCCACTggCCgCAgCCAgggC
37	gTCCCTgCCTCTATATCT	TT	CCCAgTgCCCCCCTgTTCTCCCTCC
39	gTCCCTgCCTCTATATCT	TT	TggCTgACCgCTTCACggATgCAgA

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
2	CCATggACCCgTCACTCCATgTCTT	TT	CCACTCAACTTTAACCCg
4	TCCAgCAgggATCAAgATTCATgCA	TT	CCACTCAACTTTAACCCg
6	ACgggCTATgAAATgAgAAAggCTA	TT	CCACTCAACTTTAACCCg
8	TTCACgTTTTCAgCAgACACAgTCA	TT	CCACTCAACTTTAACCCg
10	AATATTggAACCACATCTgggTgTT	TT	CCACTCAACTTTAACCCg
12	gAggCggggAgAAAAgCTATAgCgT	TT	CCACTCAACTTTAACCCg
14	TTATAAAATCCATgCAggAAggggT	TT	CCACTCAACTTTAACCCg
16	AgATggCgATAATgTgATgAACAAA	TT	CCACTCAACTTTAACCCg
18	TATATCCACgAgAgTATCTTTCCAT	TT	CCACTCAACTTTAACCCg
20	TACCCgTTAgAAggTCCCACAACAC	TT	CCACTCAACTTTAACCCg
22	CCCggCgAgAggCAgTggTggTCTT	TT	CCACTCAACTTTAACCCg
24	CCCTCgCCCTgCgTggCCTTgCCAT	TT	CCACTCAACTTTAACCCg
26	ATTCTTgTAgTgAgCCTggATAgAg	TT	CCACTCAACTTTAACCCg
28	gTgCCCATCggACATgggTgACCCT	TT	CCACTCAACTTTAACCCg
30	CTTAggggTggTgggAggAgTgggA	TT	CCACTCAACTTTAACCCg
32	TCCCCAAgggAACgCCCTTCTCgCT	TT	CCACTCAACTTTAACCCg
34	TggCCTgggTggCCggCgTgTCCgT	TT	CCACTCAACTTTAACCCg
36	CTCCATgCTgCTTggAgATCCAggC	TT	CCACTCAACTTTAACCCg
38	AAACCCTgTgAAggCTgCAgCTCCT	TT	CCACTCAACTTTAACCCg
40	AgggTCCAgTCATAgCCgCTCAgCA	TT	CCACTCAACTTTAACCCg

S4.3 Standard probes for Figures S9 and S10

Organism: ***G. gallus domesticus***
Target mRNA: **SRY (sex determining region Y)-box 10 (*Sox10*)**
Probe set: **20 probe pairs (odd probes carry full HCR initiator, even probes carry no initiator)**
HCR amplifier: **B3-Alexa647**

Odd #	Initiator I1	Spacer	Probe Sequence (25 nt)
1	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	gTCTTCCTCATCTAgAAggCCAATA
3	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	TgTgAATCTTAggCAggACTgCTgC
5	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	gCTgACAgTgCAgTTCCTgAATCCT
7	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	TgCTggAggAgCAAggACCTggTCT
9	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	CAAgTgCATggTAgCTTTCTTggTg
11	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	AgCTgTgAAAATCAgCAAggAAgCA
13	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	gggATTAAACAgATgggACAggggg
15	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	AgCAgTgATgTACACCCCATCggCC
17	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	TgAgTgAAAgTAggAgggAggTgCT
19	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	CTCTCTgATCAgTTgTCAggAgTCA
21	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	gAACCCACTgggCTCATCTCCACCT
23	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	TTCCgCCTCCgTggCTggTACTTgT
25	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	AgCCCCgCCAgCCTCCCCCTCCACC
27	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	CCCAggATgCCTgTggTCCAggTgg
29	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	gCCgTggCTCTgACCTgAAgAgTgC
31	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	gAgTCAgCTTTgCCTgCCTgCAgCT
33	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	ggTggCAggTATTggTCAAATTCgT
35	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	AgTgTCCACTggCCgCAgCCAgggC
37	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	CCCAgTgCCCCCCTgTTCTCCCTCC
39	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg	TACAA	TggCTgACCgCTTCACggATgCAgA

Even #	Probe Sequence (25 nt)
2	CCATggACCCgTCACTCCATgTCTT
4	TCCAgCAgggATCAAgATTCATgCA
6	ACgggCTATgAAATgAgAAAggCTA
8	TTCACgTTTTCAgCAgACACAgTCA
10	AATATTggAACCACATCTgggTgTT
12	gAggCggggAgAAAAgCTATAgCgT
14	TTATAAAATCCATgCAggAAggggT
16	AgATggCgATAATgTgATgAACAAA
18	TATATCCACgAgAgTATCTTTCCAT
20	TACCCgTTAgAAggTCCCACAACAC
22	CCCggCgAgAggCAgTggTggTCTT
24	CCCTCgCCCTgCgTggCCTTgCCAT
26	ATTCTTgTAgTgAgCCTggATAgAg
28	gTgCCCATCggACATgggTgACCCT
30	CTTAggggTggTgggAggAgTgggA
32	TCCCCAAgggAACgCCCTTCTCgCT
34	TggCCTgggTggCCggCgTgTCCgT
36	CTCCATgCTgCTTggAgATCCAggC
38	AAACCCTgTgAAggCTgCAgCTCCT
40	AgggTCCAgTCATAgCCgCTCAgCA

Organism: ***G. gallus domesticus***
Target mRNA: **SRY (sex determining region Y)-box 10 (*Sox10*)**
Probe set: **20 probe pairs (odd probes carry no initiator, even probes carry full HCR initiator)**
HCR amplifier: **B3-Alexa647**

Odd #	Probe Sequence (25 nt)
1	gTCTTCCTCATCTAgAAggCCAATA
3	TgTgAATCTTAggCAggACTgCTgC
5	gCTgACAgTgCAgTTCCTgAATCCT
7	TgCTggAggAgCAAggACCTggTCT
9	CAAgTgCATggTAgCTTTCTTggTg
11	AgCTgTgAAAATCAgCAAggAAgCA
13	gggATTAAACAgATgggACAggggg
15	AgCAgTgATgTACACCCCATCggCC
17	TgAgTgAAAgTAggAgggAggTgCT
19	CTCTCTgATCAgTTgTCAggAgTCA
21	gAACCCACTgggCTCATCTCCACCT
23	TTCCgCCTCCgTggCTggTACTTgT
25	AgCCCCgCCAgCCTCCCCCTCCACC
27	CCCAggATgCCTgTggTCCAggTgg
29	gCCgTggCTCTgACCTgAAgAgTgC
31	gAgTCAgCTTTgCCTgCCTgCAgCT
33	ggTggCAggTATTggTCAAATTCgT
35	AgTgTCCACTggCCgCAgCCAgggC
37	CCCAgTgCCCCCCTgTTCTCCCTCC
39	TggCTgACCgCTTCACggATgCAgA

Even #	Probe Sequence (25 nt)	Spacer	Initiator I1
2	CCATggACCCgTCACTCCATgTCTT	TACTT	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg
4	TCCAgCAgggATCAAgATTCATgCA	TACTT	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg
6	ACgggCTATgAAATgAgAAAggCTA	TACTT	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg
8	TTCACgTTTTCAgCAgACACAgTCA	TACTT	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg
10	AATATTggAACCACATCTgggTgTT	TACTT	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg
12	gAggCggggAgAAAAgCTATAgCgT	TACTT	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg
14	TTATAAAATCCATgCAggAAggggT	TACTT	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg
16	AgATggCgATAATgTgATgAACAAA	TACTT	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg
18	TATATCCACgAgAgTATCTTTCCAT	TACTT	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg
20	TACCCgTTAgAAggTCCCACAACAC	TACTT	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg
22	CCCggCgAgAggCAgTggTggTCTT	TACTT	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg
24	CCCTCgCCCTgCgTggCCTTgCCAT	TACTT	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg
26	ATTCTTgTAgTgAgCCTggATAgAg	TACTT	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg
28	gTgCCCATCggACATgggTgACCCT	TACTT	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg
30	CTTAggggTggTgggAggAgTgggA	TACTT	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg
32	TCCCCAAgggAACgCCCTTCTCgCT	TACTT	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg
34	TggCCTgggTggCCggCgTgTCCgT	TACTT	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg
36	CTCCATgCTgCTTggAgATCCAggC	TACTT	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg
38	AAACCCTgTgAAggCTgCAgCTCCT	TACTT	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg
40	AgggTCCAgTCATAgCCgCTCAgCA	TACTT	gTCCCTgCCTCTATATCTCCACTCAACTTTAACCCg

S4.4 Split-initiator probes for Figure S11

Organism: ***G. gallus domesticus***
Target mRNA: **EPH receptor A4 (*EphA4*)**
Probe set: **20 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B2-Alexa647**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
1	CCTCgTAAATCCTCATCA	AA	gCgCCgACggggACCCCggCCATgC
5	CCTCgTAAATCCTCATCA	AA	CCCAgCTCCCCCTgCACCgAgCggg
9	CCTCgTAAATCCTCATCA	AA	TgACTgggCTCCATCACATTgCAAA
13	CCTCgTAAATCCTCATCA	AA	TCCTTgTCgTTgTTTgATTCATAgT
17	CCTCgTAAATCCTCATCA	AA	AgTgggCACTTCTTATAgAAgACAC
21	CCTCgTAAATCCTCATCA	AA	ACgTCCTTCTCTTCCgAgTTgTTgA
25	CCTCgTAAATCCTCATCA	AA	ATTTTgCAAgCTTggCATTCACCAT
29	CCTCgTAAATCCTCATCA	AA	AAgAAgCCCCgATCACAggTgCAgg
33	CCTCgTAAATCCTCATCA	AA	gCgCTCCACTCCAAgTTCACTgACg
37	CCTCgTAAATCCTCATCA	AA	AAATgTACACCACTgCCACAggACC
41	CCTCgTAAATCCTCATCA	AA	TgCTTggACACTCCATTCACTgCCC
45	CCTCgTAAATCCTCATCA	AA	gCAACgCTgTgCCTCgTTATCTCTT
49	CCTCgTAAATCCTCATCA	AA	gCTgTCTTCACAATgCgATAgCTgC
53	CCTCgTAAATCCTCATCA	AA	AACTCAAACggCCCACTgAAgTCTC
57	CCTCgTAAATCCTCATCA	AA	gCTgCAATgAgAATgACCACgAgAA
61	CCTCgTAAATCCTCATCA	AA	TATgTAAAAggATCCACATATgTTC
65	CCTCgTAAATCCTCATCA	AA	CATTTAgTAACAACgCCTTCCAAgT
69	CCTCgTAAATCCTCATCA	AA	TCCCgATgCACATAgCTCATgTCAg
73	CCTCgTAAATCCTCATCA	AA	CCAAATTTAggTCTgTCgCTgCgTT
77	CCTCgTAAATCCTCATCA	AA	ggggAgCTgggATCCAgCAgggCTg

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
2	CAgACgCCgACgAggAgCgggAggA	AA	ATCATCCAgTAAACCgCC
6	CCTCCTTCCAgCgggCTCgCgATCC	AA	ATCATCCAgTAAACCgCC
10	ATCCAATCAgTTCgTAACCAATTAT	AA	ATCATCCAgTAAACCgCC
14	gCAAACTggCTCTCTCgAATAAAAC	AA	ATCATCCAgTAAACCgCC
18	ggAAACTgTgCCAggTTTCgAACTg	AA	ATCATCCAgTAAACCgCC
22	CCATCTgCCCCgCAgTACATTTTTg	AA	ATCATCCAgTAAACCgCC
26	TCTgTTgAgAgCgCCTTgTAgTATC	AA	ATCATCCAgTAAACCgCC
30	ATggATgCAgCATCATTTTCTgCTC	AA	ATCATCCAgTAAACCgCC
34	TCgTCCCgTCCTCCCTTgTTCTgTg	AA	ATCATCCAgTAAACCgCC
38	gTTTTCAgCCCgTTCTgCTgggggC	AA	ATCATCCAgTAAACCgCC
42	gACACAgCTTggTCCTggCTggggT	AA	ATCATCCAgTAAACCgCC
46	ggCCTgTCAggTTCCAgCCAggCCA	AA	ATCATCCAgTAAACCgCC
50	AAACCTTTgATgTCAgTATTCCTgg	AA	ATCATCCAgTAAACCgCC
54	ATgggggAAggAACTgTgTTAgTTg	AA	ATCATCCAgTAAACCgCC
58	TTgCTgCgCCTCCTgCTgATgACAA	AA	ATCATCCAgTAAACCgCC
62	TCCCTCACAgCTTgATTTggATCCT	AA	ATCATCCAgTAAACCgCC
66	TACTCAgTTATgATCATTACTggTT	AA	ATCATCCAgTAAACCgCC
70	TTgACCAgTATgTTTCgAgCAgCTA	AA	ATCATCCAgTAAACCgCC
74	AgTTTgTCCAgCATgTTgACAATCT	AA	ATCATCCAgTAAACCgCC
78	CTgACAgAAACAACCgCCgAgAACT	AA	ATCATCCAgTAAACCgCC

S4.5 Split-initiator probes for Figures 4 and S12

Organism: ***G. gallus domesticus***
Target mRNA: **forkhead box D3 (*FoxD3*)**
Probe set: **12 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B4-Alexa488**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
1	CCTCAACCTACCTCCAAC	AA	CgCCgCCCgATAgAgTCATCCCCgC
3	CCTCAACCTACCTCCAAC	AA	CgTCgATATCCACgTCCTCggCCgC
5	CCTCAACCTACCTCCAAC	AA	CCCgCAggTgCTCCTgCTggTgCCg
7	CCTCAACCTACCTCCAAC	AA	AgggCCCggCCAgCCCgTAggCgCC
9	CCTCAACCTACCTCCAAC	AA	gCAgAgCggCggggTgCgggTAggC
11	CCTCAACCTACCTCCAAC	AA	gCAgCgggCACgCgggCggCAgCAT
13	CCTCAACCTACCTCCAAC	AA	ggCTgggCCCgAgCTgCgCgTTgAA
15	CCTCAACCTACCTCCAAC	AA	gCTCggATTTCACgATggAgCCCgC
17	CCTCAACCTACCTCCAAC	AA	TggCggCggggCCgCCgATgATgTT
19	CCTCAACCTACCTCCAAC	AA	CCgACTgCACggTgACgggCggCCg
21	CCTCAACCTACCTCCAAC	AA	gCgCgATggCCgCggTggTCCTggC
23	CCTCAACCTACCTCCAAC	AA	CCgTTTCCCAgAgATACgTCCgggg

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
2	gCgCggTCTggCCggACATATCgCT	AT	TCTCACCATATTCgCTTC
4	CCggCgCgTCgTCTCCCTCgCCCAC	AT	TCTCACCATATTCgCTTC
6	AgCCCTgCATCATgAgCgCCgTCTg	AT	TCTCACCATATTCgCTTC
8	CgggggCAgCCCgTAggggCggCC	AT	TCTCACCATATTCgCTTC
10	gCCCgACgggCgggATgTAggggTA	AT	TCTCACCATATTCgCTTC
12	CTTTgCggCTCAgCTCgCCCgACgg	AT	TCTCACCATATTCgCTTC
14	CCCCCAAACTgCTgAgCTgCAgCTg	AT	TCTCACCATATTCgCTTC
16	CgATgCTgAACgAggggCggCTgCT	AT	TCTCACCATATTCgCTTC
18	ggAAAgTCTgCgCgCTgggCgCCgA	AT	TCTCACCATATTCgCTTC
20	ACgCCAgCggCTggTgCgCCACCAg	AT	TCTCACCATATTCgCTTC
22	TgATgTTggTAggCACgCTgAggAT	AT	TCTCACCATATTCgCTTC
24	AAATAAAAACCCCgAAAgCgACCTC	AT	TCTCACCATATTCgCTTC

Organism: ***G. gallus domesticus***
Target mRNA: **diencephalon/mesencephalon homeobox 1 (*Dmbx1*)**
Probe set: **20 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B1-Alexa514**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
1	gAggAgggCAgCAAACgg	AA	CgCTCAgTgAgTTCATggCATgCAg
5	gAggAgggCAgCAAACgg	AA	AAATgATgTCAgCCAgTCTCTCCgC
9	gAggAgggCAgCAAACgg	AA	AAATgATgTCAgCCAgTCTCTCCgC
13	gAggAgggCAgCAAACgg	AA	CAgTgTCAggTATTgTggACTgggT
17	gAggAgggCAgCAAACgg	AA	CCTCCTCTCTgTCAgTCTggTCCTC
21	gAggAgggCAgCAAACgg	AA	CgCTgATgggAgACTCTgATTTTgg
25	gAggAgggCAgCAAACgg	AA	CTgCCATgTgCTggCggAATTgCTC
29	gAggAgggCAgCAAACgg	AA	gACAgTgCAgggAgCTCAgAggCgC
33	gAggAgggCAgCAAACgg	AA	TCTCAATACTTgTTgTTTTACTgTT
37	gAggAgggCAgCAAACgg	AA	gACCTCCggTgCATCTTCTTATggg
41	gAggAgggCAgCAAACgg	AA	CggTgCTAgTAAgACATTAgTAAAT
45	gAggAgggCAgCAAACgg	AA	TCACAgCAgTCCAAAgggACAgTTC
49	gAggAgggCAgCAAACgg	AA	gTTTTCCCAAAgAATgCATCgACAA
53	gAggAgggCAgCAAACgg	AA	ggggAATAAAAgCAAAAgAggCCAC
57	gAggAgggCAgCAAACgg	AA	TTTgCTCTAAgCACCATTAAgACTC
61	gAggAgggCAgCAAACgg	AA	ggAAgTgCTTAAACAggAAATTCAC
65	gAggAgggCAgCAAACgg	AA	AATTTggCTTTCATTTTTCTCCCCA
69	gAggAgggCAgCAAACgg	AA	CTAgACCAAAATgCTCTCCAAAAAg
73	gAggAgggCAgCAAACgg	AA	TAAgAACAgCTTgCATTAATCgTgg
77	gAggAgggCAgCAAACgg	AA	CTTggCCTCCAgCATTgCAgCATTT

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
2	CTgCCTgCTggTgCAggTTgTACAT	TA	gAAgAgTCTTCCTTTACg
6	ggTgCTgggATCCATAACgTgCCTC	TA	gAAgAgTCTTCCTTTACg
10	gCTTCTgCAgCTgCTCCTTCTggAg	TA	gAAgAgTCTTCCTTTACg
14	CCTCACTTgggATgCTCTgAgTCTT	TA	gAAgAgTCTTCCTTTACg
18	TAgCCTCATCCAAggTgCTCTTAAA	TA	gAAgAgTCTTCCTTTACg
22	CACTgCTAgATgAAggAgTCACggT	TA	gAAgAgTCTTCCTTTACg
26	AggAgTAATggACCAAgTTgTTggT	TA	gAAgAgTCTTCCTTTACg
30	CgTgggAgAgAgATTggTAgTACgA	TA	gAAgAgTCTTCCTTTACg
34	CATgCTgCTTTgCCCggAgCCTTAg	TA	gAAgAgTCTTCCTTTACg
38	ggTTCCAggAgTgACATgTCTggTg	TA	gAAgAgTCTTCCTTTACg
42	AgCCAgTAgCAgTgTCTgATgCAAT	TA	gAAgAgTCTTCCTTTACg
46	gCTCTTTCTgAATgTTACAggCTTA	TA	gAAgAgTCTTCCTTTACg
50	TATgTACAAgACAAAgCAggACTCT	TA	gAAgAgTCTTCCTTTACg
54	gACTAgCTACCAAAACTgAgAgAgA	TA	gAAgAgTCTTCCTTTACg
58	gAgCAgTgAATTgCATAATggTTTT	TA	gAAgAgTCTTCCTTTACg
62	AgTAAAggAAAAAACACTTgCCTTT	TA	gAAgAgTCTTCCTTTACg
66	AACAATCAAgTCAAAAgTAACCATg	TA	gAAgAgTCTTCCTTTACg
70	AgTTTTTATTgTTCTCTATTgTCgA	TA	gAAgAgTCTTCCTTTACg
74	TAgAATTTggTgATCggAgCgTTTT	TA	gAAgAgTCTTCCTTTACg
78	AATAgAAAgCCCCCgATTATCACCC	TA	gAAgAgTCTTCCTTTACg

Organism: ***G. gallus domesticus***
Target mRNA: **SRY (sex determining region Y)-box 10 (*Sox10*)**
Probe set: **20 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B3-Alexa546**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
1	gTCCCTgCCTCTATATCT	TT	gTCTTCCTCATCTAgAAggCCAATA
3	gTCCCTgCCTCTATATCT	TT	TgTgAATCTTAggCAggACTgCTgC
5	gTCCCTgCCTCTATATCT	TT	gCTgACAgTgCAgTTCCTgAATCCT
7	gTCCCTgCCTCTATATCT	TT	TgCTggAggAgCAAggACCTggTCT
9	gTCCCTgCCTCTATATCT	TT	CAAgTgCATggTAgCTTTCTTggTg
11	gTCCCTgCCTCTATATCT	TT	AgCTgTgAAAATCAgCAAggAAgCA
13	gTCCCTgCCTCTATATCT	TT	gggATTAAACAgATgggACAggggg
15	gTCCCTgCCTCTATATCT	TT	AgCAgTgATgTACACCCCATCggCC
17	gTCCCTgCCTCTATATCT	TT	TgAgTgAAAgTAggAgggAggTgCT
19	gTCCCTgCCTCTATATCT	TT	CTCTCTgATCAgTTgTCAggAgTCA
21	gTCCCTgCCTCTATATCT	TT	gAACCCACTgggCTCATCTCCACCT
23	gTCCCTgCCTCTATATCT	TT	TTCCgCCTCCgTggCTggTACTTgT
25	gTCCCTgCCTCTATATCT	TT	AgCCCCgCCAgCCTCCCCCTCCACC
27	gTCCCTgCCTCTATATCT	TT	CCCAggATgCCTgTggTCCAggTgg
29	gTCCCTgCCTCTATATCT	TT	gCCgTggCTCTgACCTgAAgAgTgC
31	gTCCCTgCCTCTATATCT	TT	gAgTCAgCTTTgCCTgCCTgCAgCT
33	gTCCCTgCCTCTATATCT	TT	ggTggCAggTATTggTCAAATTCgT
35	gTCCCTgCCTCTATATCT	TT	AgTgTCCACTggCCgCAgCCAgggC
37	gTCCCTgCCTCTATATCT	TT	CCCAgTgCCCCCCTgTTCTCCCTCC
39	gTCCCTgCCTCTATATCT	TT	TggCTgACCgCTTCACggATgCAgA

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
2	CCATggACCCgTCACTCCATgTCTT	TT	CCACTCAACTTTAACCCg
4	TCCAgCAgggATCAAgATTCATgCA	TT	CCACTCAACTTTAACCCg
6	ACgggCTATgAAATgAgAAAggCTA	TT	CCACTCAACTTTAACCCg
8	TTCACgTTTTCAgCAgACACAgTCA	TT	CCACTCAACTTTAACCCg
10	AATATTggAACCACATCTgggTgTT	TT	CCACTCAACTTTAACCCg
12	gAggCggggAgAAAAgCTATAgCgT	TT	CCACTCAACTTTAACCCg
14	TTATAAAATCCATgCAggAAggggT	TT	CCACTCAACTTTAACCCg
16	AgATggCgATAATgTgATgAACAAA	TT	CCACTCAACTTTAACCCg
18	TATATCCACgAgAgTATCTTTCCAT	TT	CCACTCAACTTTAACCCg
20	TACCCgTTAgAAggTCCCACAACAC	TT	CCACTCAACTTTAACCCg
22	CCCggCgAgAggCAgTggTggTCTT	TT	CCACTCAACTTTAACCCg
24	CCCTCgCCCTgCgTggCCTTgCCAT	TT	CCACTCAACTTTAACCCg
26	ATTCTTgTAgTgAgCCTggATAgAg	TT	CCACTCAACTTTAACCCg
28	gTgCCCATCggACATgggTgACCCT	TT	CCACTCAACTTTAACCCg
30	CTTAggggTggTgggAggAgTgggA	TT	CCACTCAACTTTAACCCg
32	TCCCCAAgggAACgCCCTTCTCgCT	TT	CCACTCAACTTTAACCCg
34	TggCCTgggTggCCggCgTgTCCgT	TT	CCACTCAACTTTAACCCg
36	CTCCATgCTgCTTggAgATCCAggC	TT	CCACTCAACTTTAACCCg
38	AAACCCTgTgAAggCTgCAgCTCCT	TT	CCACTCAACTTTAACCCg
40	AgggTCCAgTCATAgCCgCTCAgCA	TT	CCACTCAACTTTAACCCg

Organism: ***G. gallus domesticus***
Target mRNA: **EPH receptor A4 (*EphA4*)**
Probe set: **20 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B2-Alexa647**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
1	CCTCgTAAATCCTCATCA	AA	gCgCCgACggggACCCCggCCATgC
5	CCTCgTAAATCCTCATCA	AA	CCCAgCTCCCCCTgCACCgAgCggg
9	CCTCgTAAATCCTCATCA	AA	TgACTgggCTCCATCACATTgCAAA
13	CCTCgTAAATCCTCATCA	AA	TCCTTgTCgTTgTTTgATTCATAgT
17	CCTCgTAAATCCTCATCA	AA	AgTgggCACTTCTTATAgAAgACAC
21	CCTCgTAAATCCTCATCA	AA	ACgTCCTTCTCTTCCgAgTTgTTgA
25	CCTCgTAAATCCTCATCA	AA	ATTTTgCAAgCTTggCATTCACCAT
29	CCTCgTAAATCCTCATCA	AA	AAgAAgCCCCgATCACAggTgCAgg
33	CCTCgTAAATCCTCATCA	AA	gCgCTCCACTCCAAgTTCACTgACg
37	CCTCgTAAATCCTCATCA	AA	AAATgTACACCACTgCCACAggACC
41	CCTCgTAAATCCTCATCA	AA	TgCTTggACACTCCATTCACTgCCC
45	CCTCgTAAATCCTCATCA	AA	gCAACgCTgTgCCTCgTTATCTCTT
49	CCTCgTAAATCCTCATCA	AA	gCTgTCTTCACAATgCgATAgCTgC
53	CCTCgTAAATCCTCATCA	AA	AACTCAAACggCCCACTgAAgTCTC
57	CCTCgTAAATCCTCATCA	AA	gCTgCAATgAgAATgACCACgAgAA
61	CCTCgTAAATCCTCATCA	AA	TATgTAAAAggATCCACATATgTTC
65	CCTCgTAAATCCTCATCA	AA	CATTTAgTAACAACgCCTTCCAAgT
69	CCTCgTAAATCCTCATCA	AA	TCCCgATgCACATAgCTCATgTCAg
73	CCTCgTAAATCCTCATCA	AA	CCAAATTTAggTCTgTCgCTgCgTT
77	CCTCgTAAATCCTCATCA	AA	ggggAgCTgggATCCAgCAgggCTg

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
2	CAgACgCCgACgAggAgCgggAggA	AA	ATCATCCAgTAAACCgCC
6	CCTCCTTCCAgCgggCTCgCgATCC	AA	ATCATCCAgTAAACCgCC
10	ATCCAATCAgTTCgTAACCAATTAT	AA	ATCATCCAgTAAACCgCC
14	gCAAACTggCTCTCTCgAATAAAAC	AA	ATCATCCAgTAAACCgCC
18	ggAAACTgTgCCAggTTTCgAACTg	AA	ATCATCCAgTAAACCgCC
22	CCATCTgCCCCgCAgTACATTTTTg	AA	ATCATCCAgTAAACCgCC
26	TCTgTTgAgAgCgCCTTgTAgTATC	AA	ATCATCCAgTAAACCgCC
30	ATggATgCAgCATCATTTTCTgCTC	AA	ATCATCCAgTAAACCgCC
34	TCgTCCCgTCCTCCCTTgTTCTgTg	AA	ATCATCCAgTAAACCgCC
38	gTTTTCAgCCCgTTCTgCTgggggC	AA	ATCATCCAgTAAACCgCC
42	gACACAgCTTggTCCTggCTggggT	AA	ATCATCCAgTAAACCgCC
46	ggCCTgTCAggTTCCAgCCAggCCA	AA	ATCATCCAgTAAACCgCC
50	AAACCTTTgATgTCAgTATTCCTgg	AA	ATCATCCAgTAAACCgCC
54	ATgggggAAggAACTgTgTTAgTTg	AA	ATCATCCAgTAAACCgCC
58	TTgCTgCgCCTCCTgCTgATgACAA	AA	ATCATCCAgTAAACCgCC
62	TCCCTCACAgCTTgATTTggATCCT	AA	ATCATCCAgTAAACCgCC
66	TACTCAgTTATgATCATTACTggTT	AA	ATCATCCAgTAAACCgCC
70	TTgACCAgTATgTTTCgAgCAgCTA	AA	ATCATCCAgTAAACCgCC
74	AgTTTgTCCAgCATgTTgACAATCT	AA	ATCATCCAgTAAACCgCC
78	CTgACAgAAACAACCgCCgAgAACT	AA	ATCATCCAgTAAACCgCC

S4.6 Split-initiator probes for Figures S13–S17

Organism: ***G. gallus domesticus***
Target mRNA: **EPH receptor A4 (*EphA4*)**
Probe set: **20 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B1-Alexa546**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
3	gAggAgggCAgCAAACgg	AA	TACACgCgggAgCCggTgACggCCC
7	gAggAgggCAgCAAACgg	AA	TCATCCATTATgCTCACTTCCTCCC
11	gAggAgggCAgCAAACgg	AA	TATACCCTCTgAgCCCCCTCgCggg
15	gAggAgggCAgCAAACgg	AA	ACCTCTgTATTCAgCTTCATgATCC
19	gAggAgggCAgCAAACgg	AA	gATgTATCAgCCCCAgTAATggTgT
23	gAggAgggCAgCAAACgg	AA	CAgTTgCCAATgggTACCAgCCATT
27	gAggAgggCAgCAAACgg	AA	TgAggCgggCATTTggCACATgCAA
31	gAggAgggCAgCAAACgg	AA	ggTgCggATggAgggCgAgTgCAgg
35	gAggAgggCAgCAAACgg	AA	CgCTTgCACACCACgTTgTAggAgA
39	gAggAgggCAgCAAACgg	AA	AggAggTCAgTgATggAAACCTTCg
43	gAggAgggCAgCAAACgg	AA	gCTgCTTggTTAgTTgTCACAgTgA
47	gAggAgggCAgCAAACgg	AA	ACTTCATACTCCAggATgACTCCAT
51	gAggAgggCAgCAAACgg	AA	TgAAATACATATgAAgTCAgggggT
55	gAggAgggCAgCAAACgg	AA	ACTgTgggATTggTACCATCgCCAA
59	gAggAgggCAgCAAACgg	AA	TCTgCCTCTTgCTTAgCTTTACTgT
63	gAggAgggCAgCAAACgg	AA	TTCAgAgTCTTgATAgCCACACAgA
67	gAggAgggCAgCAAACgg	AA	CgAAgCATCCCCACCAACTggATTA
71	gAggAgggCAgCAAACgg	AA	TggAgAgCAATggggCAgTCCATTg
75	gAggAgggCAgCAAACgg	AA	CTCTTCAggCTgTTAgggTTgCggA
79	gAggAgggCAgCAAACgg	AA	TCCATTTTAATggCTTggAgCCAgT

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
4	TCCAgCAgggTCACTTCgTTggCgg	TA	gAAgAgTCTTCCTTTACg
8	TggTAggTgCggATCggAgTgTTCT	TA	gAAgAgTCTTCCTTTACg
12	TCTCTCAgCgTgAACTTgATTTCAA	TA	gAAgAgTCTTCCTTTACg
16	TTCTTgCTgAgAggCCCCACgTCCC	TA	gAAgAgTCTTCCTTTACg
20	CAggAgCCACgAACCTCCACCAgAg	TA	gAAgAgTCTTCCTTTACg
24	CgTTCTTCATAgCCAgCATTgCACA	TA	gAAgAgTCTTCCTTTACg
28	gTAgAgCCTTCCCAgATggAgTAgC	TA	gAAgAgTCTTCCTTTACg
32	TCgTTgACgTTggAAATCAggTTCT	TA	gAAgAgTCTTCCTTTACg
36	CAgTggCTgggCTCCCCTgCCCCgC	TA	gAAgAgTCTTCCTTTACg
40	ACCTCAAAggTgTAgTTggTgTgTg	TA	gAAgAgTCTTCCTTTACg
44	gCCTggATCAATgCAATTggggATg	TA	gAAgAgTCTTCCTTTACg
48	TCgTTTTggTCCTTTTCATAgTACT	TA	gAAgAgTCTTCCTTTACg
52	TATCCTgCTgCTgTCCTggCCCgCA	TA	gAAgAgTCTTCCTTTACg
56	ACACTgCCAgCCACTgAAACAAgCA	TA	gAAgAgTCTTCCTTTACg
60	ACACCTTggTTCAAATgTTTCTCCT	TA	gAAgAgTCTTCCTTTACg
64	CTCCgTTgTTTgTCAgTgTAACCAg	TA	gAAgAgTCTTCCTTTACg
68	AgATACTTCATTCCTgAgCCgATgC	TA	gAAgAgTCTTCCTTTACg
72	TTCTgCCAgCAgTCTAACATCAgCT	TA	gAAgAgTCTTCCTTTACg
76	CTgggTCTggAgCTCTCgCTgCCTg	TA	gAAgAgTCTTCCTTTACg
80	gCAgCTgTgAAgTTATCCTTgTATC	TA	gAAgAgTCTTCCTTTACg

Organism: ***G. gallus domesticus***
Target mRNA: **early growth response 2 (*Egr2*)**
Probe set: **20 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B3-Alexa647**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
1	gTCCCTgCCTCTATATCT	TT	gCAgTgCTgggCTgCTTgCAgCTgT
3	gTCCCTgCCTCTATATCT	TT	gTCTggCggTAACTATTTATgggggg
5	gTCCCTgCCTCTATATCT	TT	CACgCCgCTCATCTggTCgAACggC
7	gTCCCTgCCTCTATATCT	TT	AACCAACCCCAACCAgTgCgTACAA
9	gTCCCTgCCTCTATATCT	TT	CCACCCCCCCCCCCCgCAgACgCAA
11	gTCCCTgCCTCTATATCT	TT	ggTgTCAgCgTggAATAATTAAgAg
13	gTCCCTgCCTCTATATCT	TT	TgCACTTgAgTAgCTgAgAgCCTgA
15	gTCCCTgCCTCTATATCT	TT	TTTTgCCTggCAgCCCAAATggTgC
17	gTCCCTgCCTCTATATCT	TT	CgACCAgTCATTACTTTCCTCCgCA
19	gTCCCTgCCTCTATATCT	TT	ATATAAggACTgAggAACggggCCC
21	gTCCCTgCCTCTATATCT	TT	CgCgCCCTTgCgCTCCTTCTggCgC
23	gTCCCTgCCTCTATATCT	TT	ACCCCCCACATgCAgCCgggTACgg
25	gTCCCTgCCTCTATATCT	TT	ggCAAACTTTCCggTCTCggCgTCg
27	gTCCCTgCCTCTATATCT	TT	CgCTAAgATgAggggAggCgAAAgC
29	gTCCCTgCCTCTATATCT	TT	CgCTgTCCCTTCgggAgCCTgggAA
31	gTCCCTgCCTCTATATCT	TT	gAggTTgTgCTCCgCggCCgAgACA
33	gTCCCTgCCTCTATATCT	TT	gTCgCggCCAggCCgATgTgCgggg
35	gTCCCTgCCTCTATATCT	TT	AgCCATggTTATCCAAggCTgTggC
37	gTCCCTgCCTCTATATCT	TT	CTCACCACgACggCACCgCATgCAT
39	gTCCCTgCCTCTATATCT	TT	gAgCgCCgACCCTgCCgTCgAAggg

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
2	CgTgCCAgggCTgTgCCAgggCTCA	TT	CCACTCAACTTTAACCCg
4	CCCgCCgCAgCTCgCgCTggAggAC	TT	CCACTCAACTTTAACCCg
6	gTCCACgCCgAgCATgCCgTCTgCg	TT	CCACTCAACTTTAACCCg
8	CTCTTCggTCACCgTAAAgACAAAA	TT	CCACTCAACTTTAACCCg
10	gTgCgCTCgTCCAgCCCgggCCCTT	TT	CCACTCAACTTTAACCCg
12	CCCCCggCAggCACCTACggAAATA	TT	CCACTCAACTTTAACCCg
14	ggAggCCgCgAgCAgAgCCTTggCT	TT	CCACTCAACTTTAACCCg
16	TgTgACAAgTgTggTAACgCgACTT	TT	CCACTCAACTTTAACCCg
18	CAgAATAAATACgggATATCTCACC	TT	CCACTCAACTTTAACCCg
20	CCCTgAATgCCCgggACgTCACTgC	TT	CCACTCAACTTTAACCCg
22	TCCgggCTgCggggCggCggCggTC	TT	CCACTCAACTTTAACCCg
24	gCTgTCgTCggAgCggATCggTAAg	TT	CCACTCAACTTTAACCCg
26	ACAgCCATACTACAACCAgggAggg	TT	CCACTCAACTTTAACCCg
28	TgTgggACCAggTggCAAAgCTgCC	TT	CCACTCAACTTTAACCCg
30	CCATgTgCCACCTCTCCggCAgACg	TT	CCACTCAACTTTAACCCg
32	TgCCgACTgAggACTCCACgACTCT	TT	CCACTCAACTTTAACCCg
34	AAAAAAAAAAAAggAgAAAAAAgCA	TT	CCACTCAACTTTAACCCg
36	TgATgCACgACgCTCCggCTgTgAC	TT	CCACTCAACTTTAACCCg
38	TCACACCACACAAggCACCAAggAC	TT	CCACTCAACTTTAACCCg
40	TTATTCgTCgTCgCTTATAAACgCC	TT	CCACTCAACTTTAACCCg

S4.7 Split-initiator probes Figures 5, S18, and S19

Organism: ***G. gallus domesticus***
Target mRNA: **diencephalon/mesencephalon homeobox 1 (*Dmbx1*)**
Probe set: **20 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B1-Alexa546**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
1	gAggAgggCAgCAAACgg	AA	CgCTCAgTgAgTTCATggCATgCAg
5	gAggAgggCAgCAAACgg	AA	AAATgATgTCAgCCAgTCTCTCCgC
9	gAggAgggCAgCAAACgg	AA	AAATgATgTCAgCCAgTCTCTCCgC
13	gAggAgggCAgCAAACgg	AA	CAgTgTCAggTATTgTggACTgggT
17	gAggAgggCAgCAAACgg	AA	CCTCCTCTCTgTCAgTCTggTCCTC
21	gAggAgggCAgCAAACgg	AA	CgCTgATgggAgACTCTgATTTTgg
25	gAggAgggCAgCAAACgg	AA	CTgCCATgTgCTggCggAATTgCTC
29	gAggAgggCAgCAAACgg	AA	gACAgTgCAgggAgCTCAgAggCgC
33	gAggAgggCAgCAAACgg	AA	TCTCAATACTTgTTgTTTTACTgTT
37	gAggAgggCAgCAAACgg	AA	gACCTCCggTgCATCTTCTTATggg
41	gAggAgggCAgCAAACgg	AA	CggTgCTAgTAAgACATTAgTAAAT
45	gAggAgggCAgCAAACgg	AA	TCACAgCAgTCCAAAgggACAgTTC
49	gAggAgggCAgCAAACgg	AA	gTTTTCCCAAAgAATgCATCgACAA
53	gAggAgggCAgCAAACgg	AA	ggggAATAAAAgCAAAAgAggCCAC
57	gAggAgggCAgCAAACgg	AA	TTTgCTCTAAgCACCATTAAgACTC
61	gAggAgggCAgCAAACgg	AA	ggAAgTgCTTAAACAggAAATTCAC
65	gAggAgggCAgCAAACgg	AA	AATTTggCTTTCATTTTTCTCCCCA
69	gAggAgggCAgCAAACgg	AA	CTAgACCAAAATgCTCTCCAAAAAg
73	gAggAgggCAgCAAACgg	AA	TAAgAACAgCTTgCATTAATCgTgg
77	gAggAgggCAgCAAACgg	AA	CTTggCCTCCAgCATTgCAgCATTT

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
2	CTgCCTgCTggTgCAggTTgTACAT	TA	gAAgAgTCTTCCTTTACg
6	ggTgCTgggATCCATAACgTgCCTC	TA	gAAgAgTCTTCCTTTACg
10	gCTTCTgCAgCTgCTCCTTCTggAg	TA	gAAgAgTCTTCCTTTACg
14	CCTCACTTgggATgCTCTgAgTCTT	TA	gAAgAgTCTTCCTTTACg
18	TAgCCTCATCCAAggTgCTCTTAAA	TA	gAAgAgTCTTCCTTTACg
22	CACTgCTAgATgAAggAgTCACggT	TA	gAAgAgTCTTCCTTTACg
26	AggAgTAATggACCAAgTTgTTggT	TA	gAAgAgTCTTCCTTTACg
30	CgTgggAgAgAgATTggTAgTACgA	TA	gAAgAgTCTTCCTTTACg
34	CATgCTgCTTTgCCCggAgCCTTAg	TA	gAAgAgTCTTCCTTTACg
38	ggTTCCAggAgTgACATgTCTggTg	TA	gAAgAgTCTTCCTTTACg
42	AgCCAgTAgCAgTgTCTgATgCAAT	TA	gAAgAgTCTTCCTTTACg
46	gCTCTTTCTgAATgTTACAggCTTA	TA	gAAgAgTCTTCCTTTACg
50	TATgTACAAgACAAAgCAggACTCT	TA	gAAgAgTCTTCCTTTACg
54	gACTAgCTACCAAAACTgAgAgAgA	TA	gAAgAgTCTTCCTTTACg
58	gAgCAgTgAATTgCATAATggTTTT	TA	gAAgAgTCTTCCTTTACg
62	AgTAAAggAAAAAACACTTgCCTTT	TA	gAAgAgTCTTCCTTTACg
66	AACAATCAAgTCAAAAgTAACCATg	TA	gAAgAgTCTTCCTTTACg
70	AgTTTTTATTgTTCTCTATTgTCgA	TA	gAAgAgTCTTCCTTTACg
74	TAgAATTTggTgATCggAgCgTTTT	TA	gAAgAgTCTTCCTTTACg
78	AATAgAAAgCCCCCgATTATCACCC	TA	gAAgAgTCTTCCTTTACg

Organism: ***G. gallus domesticus***
Target mRNA: **diencephalon/mesencephalon homeobox 1 (*Dmbx1*)**
Probe set: **20 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B2-Alexa647**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
3	CCTCgTAAATCCTCATCA	AA	AgTCgggCgCgTgCTgTgCTTgCTg
7	CCTCgTAAATCCTCATCA	AA	AggCCgTgCgACTTCgCCTTTgTTT
11	CCTCgTAAATCCTCATCA	AA	CACTgTggCTgCTTTCACAgTCCTT
15	CCTCgTAAATCCTCATCA	AA	ACAgggTTAggTTgAggTCTgTgCT
19	CCTCgTAAATCCTCATCA	AA	CgTCCACACCAggACTCTTgTCCAC
23	CCTCgTAAATCCTCATCA	AA	AggAgTAggAgTgAgTTTgAgCCAg
27	CCTCgTAAATCCTCATCA	AA	TggCAgACggTgTCCCCATTTCgAA
31	CCTCgTAAATCCTCATCA	AA	ggATgggTgAggACCAgACCTgCTg
35	CCTCgTAAATCCTCATCA	AA	gTAgggTgTCAAgCCCAAgggATgC
39	CCTCgTAAATCCTCATCA	AA	ACTCCAggAAgAgATgAgggTggAA
43	CCTCgTAAATCCTCATCA	AA	TCCTCCTCAAATATTTAAAgAAgAC
47	CCTCgTAAATCCTCATCA	AA	ACATTATCgCAgggATgAggTgAgg
51	CCTCgTAAATCCTCATCA	AA	gCggTggATgCTTTCAACATTgTAA
55	CCTCgTAAATCCTCATCA	AA	gggAgCgTggCTgATTTgTgACTTT
59	CCTCgTAAATCCTCATCA	AA	TCAgCTTTAgCAgAgAAgAgAgAAg
63	CCTCgTAAATCCTCATCA	AA	TCTgTCTgTgAACAAgTgCTATTAg
67	CCTCgTAAATCCTCATCA	AA	TTACACTTCACTgAAgACCAAAgAg
71	CCTCgTAAATCCTCATCA	AA	gTATgAACACAgTgggAgTTCATAC
75	CCTCgTAAATCCTCATCA	AA	AgggTCTgAAgCTgCACAgCTTgAg
79	CCTCgTAAATCCTCATCA	AA	CAAgCCAATCTACTCCTCgCTgCAg

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
4	gggTgAgggCgTgCACTgAgggCCg	AA	ATCATCCAgTAAACCgCC
8	CCAgTgCCTCCAgCTgTTgggCAgT	AA	ATCATCCAgTAAACCgCC
12	CCAgCACAggTggTTCCgTCTTCCC	AA	ATCATCCAgTAAACCgCC
16	gCgCTgATTCACTggCTgACTgCTC	AA	ATCATCCAgTAAACCgCC
20	TCgCTCTCTTgCAgTTCAAAgCCTT	AA	ATCATCCAgTAAACCgCC
24	gCAggCggAAgAggCTCAggggTgA	AA	ATCATCCAgTAAACCgCC
28	TgTTgACATTCATgCCCAAgTAggg	AA	ATCATCCAgTAAACCgCC
32	ggCTTggAAgAgAgCTggAggCCTg	AA	ATCATCCAgTAAACCgCC
36	ACggTTgCCCATCTggCAgTCAgTT	AA	ATCATCCAgTAAACCgCC
40	AAAgTTTTCCCTgATAgggAgCACC	AA	ATCATCCAgTAAACCgCC
44	CTgTCTAAACACACATCCTCTCCCT	AA	ATCATCCAgTAAACCgCC
48	AAAAAgggTgTATATAACACggTTg	AA	ATCATCCAgTAAACCgCC
52	TTCTgTAACACTgACAgTAACACAC	AA	ATCATCCAgTAAACCgCC
56	AACCCAAgAAgAgCAACTAgCTgTg	AA	ATCATCCAgTAAACCgCC
60	TTgCATCATTTCCTggCCgTTATAA	AA	ATCATCCAgTAAACCgCC
64	CAgCAgCATTTggCCAgCATTTTgT	AA	ATCATCCAgTAAACCgCC
68	AACCCCATAATTTgTAAATgggggA	AA	ATCATCCAgTAAACCgCC
72	TTgTCAAggAACCATATAATTCATg	AA	ATCATCCAgTAAACCgCC
76	CACTTgTTACATTCTCACTTgCTAA	AA	ATCATCCAgTAAACCgCC
80	ggTTgCTTggggACATggTACTTTT	AA	ATCATCCAgTAAACCgCC

Organism: ***G. gallus domesticus***
Target mRNA: **EPH receptor A4 (*EphA4*)**
Probe set: **20 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B1-Alexa546**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
3	gAggAgggCAgCAAACgg	AA	TACACgCgggAgCCggTgACggCCC
7	gAggAgggCAgCAAACgg	AA	TCATCCATTATgCTCACTTCCTCCC
11	gAggAgggCAgCAAACgg	AA	TATACCCTCTgAgCCCCCTCgCggg
15	gAggAgggCAgCAAACgg	AA	ACCTCTgTATTCAgCTTCATgATCC
19	gAggAgggCAgCAAACgg	AA	gATgTATCAgCCCCAgTAATggTgT
23	gAggAgggCAgCAAACgg	AA	CAgTTgCCAATgggTACCAgCCATT
27	gAggAgggCAgCAAACgg	AA	TgAggCgggCATTTggCACATgCAA
31	gAggAgggCAgCAAACgg	AA	ggTgCggATggAgggCgAgTgCAgg
35	gAggAgggCAgCAAACgg	AA	CgCTTgCACACCACgTTgTAggAgA
39	gAggAgggCAgCAAACgg	AA	AggAggTCAgTgATggAAACCTTCg
43	gAggAgggCAgCAAACgg	AA	gCTgCTTggTTAgTTgTCACAgTgA
47	gAggAgggCAgCAAACgg	AA	ACTTCATACTCCAggATgACTCCAT
51	gAggAgggCAgCAAACgg	AA	TgAAATACATATgAAgTCAgggggT
55	gAggAgggCAgCAAACgg	AA	ACTgTgggATTggTACCATCgCCAA
59	gAggAgggCAgCAAACgg	AA	TCTgCCTCTTgCTTAgCTTTACTgT
63	gAggAgggCAgCAAACgg	AA	TTCAgAgTCTTgATAgCCACACAgA
67	gAggAgggCAgCAAACgg	AA	CgAAgCATCCCCACCAACTggATTA
71	gAggAgggCAgCAAACgg	AA	TggAgAgCAATggggCAgTCCATTg
75	gAggAgggCAgCAAACgg	AA	CTCTTCAggCTgTTAgggTTgCggA
79	gAggAgggCAgCAAACgg	AA	TCCATTTTAATggCTTggAgCCAgT

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
4	TCCAgCAgggTCACTTCgTTggCgg	TA	gAAgAgTCTTCCTTTACg
8	TggTAggTgCggATCggAgTgTTCT	TA	gAAgAgTCTTCCTTTACg
12	TCTCTCAgCgTgAACTTgATTTCAA	TA	gAAgAgTCTTCCTTTACg
16	TTCTTgCTgAgAggCCCCACgTCCC	TA	gAAgAgTCTTCCTTTACg
20	CAggAgCCACgAACCTCCACCAgAg	TA	gAAgAgTCTTCCTTTACg
24	CgTTCTTCATAgCCAgCATTgCACA	TA	gAAgAgTCTTCCTTTACg
28	gTAgAgCCTTCCCAgATggAgTAgC	TA	gAAgAgTCTTCCTTTACg
32	TCgTTgACgTTggAAATCAggTTCT	TA	gAAgAgTCTTCCTTTACg
36	CAgTggCTgggCTCCCCTgCCCCgC	TA	gAAgAgTCTTCCTTTACg
40	ACCTCAAAggTgTAgTTggTgTgTg	TA	gAAgAgTCTTCCTTTACg
44	gCCTggATCAATgCAATTggggATg	TA	gAAgAgTCTTCCTTTACg
48	TCgTTTTggTCCTTTTCATAgTACT	TA	gAAgAgTCTTCCTTTACg
52	TATCCTgCTgCTgTCCTggCCCgCA	TA	gAAgAgTCTTCCTTTACg
56	ACACTgCCAgCCACTgAAACAAgCA	TA	gAAgAgTCTTCCTTTACg
60	ACACCTTggTTCAAATgTTTCTCCT	TA	gAAgAgTCTTCCTTTACg
64	CTCCgTTgTTTgTCAgTgTAACCAg	TA	gAAgAgTCTTCCTTTACg
68	AgATACTTCATTCCTgAgCCgATgC	TA	gAAgAgTCTTCCTTTACg
72	TTCTgCCAgCAgTCTAACATCAgCT	TA	gAAgAgTCTTCCTTTACg
76	CTgggTCTggAgCTCTCgCTgCCTg	TA	gAAgAgTCTTCCTTTACg
80	gCAgCTgTgAAgTTATCCTTgTATC	TA	gAAgAgTCTTCCTTTACg

Organism: ***G. gallus domesticus***
Target mRNA: **EPH receptor A4 (*EphA4*)**
Probe set: **20 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B2-Alexa647**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
1	CCTCgTAAATCCTCATCA	AA	gCgCCgACggggACCCCggCCATgC
5	CCTCgTAAATCCTCATCA	AA	CCCAgCTCCCCCTgCACCgAgCggg
9	CCTCgTAAATCCTCATCA	AA	TgACTgggCTCCATCACATTgCAAA
13	CCTCgTAAATCCTCATCA	AA	TCCTTgTCgTTgTTTgATTCATAgT
17	CCTCgTAAATCCTCATCA	AA	AgTgggCACTTCTTATAgAAgACAC
21	CCTCgTAAATCCTCATCA	AA	ACgTCCTTCTCTTCCgAgTTgTTgA
25	CCTCgTAAATCCTCATCA	AA	ATTTTgCAAgCTTggCATTCACCAT
29	CCTCgTAAATCCTCATCA	AA	AAgAAgCCCCgATCACAggTgCAgg
33	CCTCgTAAATCCTCATCA	AA	gCgCTCCACTCCAAgTTCACTgACg
37	CCTCgTAAATCCTCATCA	AA	AAATgTACACCACTgCCACAggACC
41	CCTCgTAAATCCTCATCA	AA	TgCTTggACACTCCATTCACTgCCC
45	CCTCgTAAATCCTCATCA	AA	gCAACgCTgTgCCTCgTTATCTCTT
49	CCTCgTAAATCCTCATCA	AA	gCTgTCTTCACAATgCgATAgCTgC
53	CCTCgTAAATCCTCATCA	AA	AACTCAAACggCCCACTgAAgTCTC
57	CCTCgTAAATCCTCATCA	AA	gCTgCAATgAgAATgACCACgAgAA
61	CCTCgTAAATCCTCATCA	AA	TATgTAAAAggATCCACATATgTTC
65	CCTCgTAAATCCTCATCA	AA	CATTTAgTAACAACgCCTTCCAAgT
69	CCTCgTAAATCCTCATCA	AA	TCCCgATgCACATAgCTCATgTCAg
73	CCTCgTAAATCCTCATCA	AA	CCAAATTTAggTCTgTCgCTgCgTT
77	CCTCgTAAATCCTCATCA	AA	ggggAgCTgggATCCAgCAgggCTg

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
2	CAgACgCCgACgAggAgCgggAggA	AA	ATCATCCAgTAAACCgCC
6	CCTCCTTCCAgCgggCTCgCgATCC	AA	ATCATCCAgTAAACCgCC
10	ATCCAATCAgTTCgTAACCAATTAT	AA	ATCATCCAgTAAACCgCC
14	gCAAACTggCTCTCTCgAATAAAAC	AA	ATCATCCAgTAAACCgCC
18	ggAAACTgTgCCAggTTTCgAACTg	AA	ATCATCCAgTAAACCgCC
22	CCATCTgCCCCgCAgTACATTTTTg	AA	ATCATCCAgTAAACCgCC
26	TCTgTTgAgAgCgCCTTgTAgTATC	AA	ATCATCCAgTAAACCgCC
30	ATggATgCAgCATCATTTTCTgCTC	AA	ATCATCCAgTAAACCgCC
34	TCgTCCCgTCCTCCCTTgTTCTgTg	AA	ATCATCCAgTAAACCgCC
38	gTTTTCAgCCCgTTCTgCTgggggC	AA	ATCATCCAgTAAACCgCC
42	gACACAgCTTggTCCTggCTggggT	AA	ATCATCCAgTAAACCgCC
46	ggCCTgTCAggTTCCAgCCAggCCA	AA	ATCATCCAgTAAACCgCC
50	AAACCTTTgATgTCAgTATTCCTgg	AA	ATCATCCAgTAAACCgCC
54	ATgggggAAggAACTgTgTTAgTTg	AA	ATCATCCAgTAAACCgCC
58	TTgCTgCgCCTCCTgCTgATgACAA	AA	ATCATCCAgTAAACCgCC
62	TCCCTCACAgCTTgATTTggATCCT	AA	ATCATCCAgTAAACCgCC
66	TACTCAgTTATgATCATTACTggTT	AA	ATCATCCAgTAAACCgCC
70	TTgACCAgTATgTTTCgAgCAgCTA	AA	ATCATCCAgTAAACCgCC
74	AgTTTgTCCAgCATgTTgACAATCT	AA	ATCATCCAgTAAACCgCC
78	CTgACAgAAACAACCgCCgAgAACT	AA	ATCATCCAgTAAACCgCC

S4.8 Split-initiator probes for Figure 6, S20–S28

Organism: ***H. sapiens sapiens***
Target mRNA: **destabilized enhanced green fluorescent protein (*Tg(d2eGFP)*)**
Probe set: **12 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B3-Alexa594 (Figures 6A and S20)**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
1	gTCCCTgCCTCTATATCT	TT	TTgTggCCgTTTACgTCgCCgTCCA
3	gTCCCTgCCTCTATATCT	TT	AgggTCAgCTTgCCgTAggTggCAT
5	gTCCCTgCCTCTATATCT	TT	gTCACgAgggTgggCCAgggCACgg
7	gTCCCTgCCTCTATATCT	TT	TgCTTCATgTggTCggggTAgCggC
9	gTCCCTgCCTCTATATCT	TT	ATggTgCgCTCCTggACgTAgCCTT
11	gTCCCTgCCTCTATATCT	TT	CCCTCgAACTTCACCTCggCgCggg
13	gTCCCTgCCTCTATATCT	TT	CCgTCCTCCTTgAAgTCgATgCCCT
15	gTCCCTgCCTCTATATCT	TT	ATATAgACgTTgTggCTgTTgTAgT
17	gTCCCTgCCTCTATATCT	TT	TTgTggCggATCTTgAAgTTCACCT
19	gTCCCTgCCTCTATATCT	TT	ATgggggTgTTCTgCTggTAgTggT
21	gTCCCTgCCTCTATATCT	TT	gCggACTgggTgCTCAggTAgTggT
23	gTCCCTgCCTCTATATCT	TT	ACgAACTCCAgCAggACCATgTgAT

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
2	CCCTCgCCCTCgCCggACACgCTgA	TT	CCACTCAACTTTAACCCg
4	AgCTTgCCggTggTgCAgATgAACT	TT	CCACTCAACTTTAACCCg
6	AAgCACTgCACgCCgTAggTCAggg	TT	CCACTCAACTTTAACCCg
8	ggCATggCggACTTgAAgAAgTCgT	TT	CCACTCAACTTTAACCCg
10	TTgTAgTTgCCgTCgTCCTTgAAgA	TT	CCACTCAACTTTAACCCg
12	AgCTCgATgCggTTCACCAgggTgT	TT	CCACTCAACTTTAACCCg
14	TACTCCAgCTTgTgCCCCAggATgT	TT	CCACTCAACTTTAACCCg
16	ATgCCgTTCTTCTgCTTgTCggCCA	TT	CCACTCAACTTTAACCCg
18	gCgAgCTgCACgCTgCCgTCCTCgA	TT	CCACTCAACTTTAACCCg
20	TCgggCAgCAgCACggggCCgTCgC	TT	CCACTCAACTTTAACCCg
22	CgCTTCTCgTTggggTCTTTgCTCA	TT	CCACTCAACTTTAACCCg
24	ATgCCgAgAgTgATCCCggCggCgg	TT	CCACTCAACTTTAACCCg

Organism: ***H. sapiens sapiens***
Target mRNA: **glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)**
Probe set: **10 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B5-Alexa488 (Figures 6B, S24, and S23)**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
3	CTCACTCCCAATCTCTAT	AA	gggTCATTgATggCAACAATATCCA
7	CTCACTCCCAATCTCTAT	AA	TTTCCATTgATgACAAgCTTCCCgT
11	CTCACTCCCAATCTCTAT	AA	gCCTTCTCCATggTggTgAAgACgC
15	CTCACTCCCAATCTCTAT	AA	AggCTgTTgTCATACTTCTCATggT
19	CTCACTCCCAATCTCTAT	AA	gCATggACTgTggTCATgAgTCCTT
23	CTCACTCCCAATCTCTAT	AA	gCCTTggCAgCgCCAgTAgAggCAg
27	CTCACTCCCAATCTCTAT	AA	ggTTTTTCTAgACggCAggTCAggT
31	CTCACTCCCAATCTCTAT	AA	CTgTTgAAgTCAgAggAgACCACCT
35	CTCACTCCCAATCTCTAT	AA	CTggTggTCCAggggTCTTACTCCT
39	CTCACTCCCAATCTCTAT	AA	CTACATggCAACTgTgAggAggggA

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
4	TAAACCATgTAgTTgAggTCAATgA	AA	CTACCCTACAAATCCAAT
8	TCTCgCTCCTggAAgATggTgATgg	AA	CTACCCTACAAATCCAAT
12	TTggCTCCCCCCTgCAAATgAgCCC	AA	CTACCCTACAAATCCAAT
16	gTgCAggAggCATTgCTgATgATCT	AA	CTACCCTACAAATCCAAT
20	TCCACAgTCTTCTgggTggCAgTgA	AA	CTACCCTACAAATCCAAT
24	TTCAgCTCAgggATgACCTTgCCCA	AA	CTACCCTACAAATCCAAT
28	ACCTTCTTgATgTCATCATATTTgg	AA	CTACCCTACAAATCCAAT
32	gCgTCAAAggTggAggAgTgggTgT	AA	CTACCCTACAAATCCAAT
36	TCTCTTCCTCTTgTgCTCTTgCTgg	AA	CTACCCTACAAATCCAAT
40	CCTAggCCCCTCCCCTCTTCAAggg	AA	CTACCCTACAAATCCAAT

Organism: ***H. sapiens sapiens***
Target mRNA: **glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)**
Probe set: **10 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B4-Alexa594 (Figures 6B and S24), B4-Alexa488 (Figures 6C and S27)**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
1	CCTCAACCTACCTCCAAC	AA	ACCAggCgCCCAATACgACCAAATC
5	CCTCAACCTACCTCCAAC	AA	CCATgggTggAATCATATTggAACA
9	CCTCAACCTACCTCCAAC	AA	gCATCgCCCCACTTgATTTTggAgg
13	CCTCAACCTACCTCCAAC	AA	gCAgAgggggCAgAgATgATgACCC
17	CCTCAACCTACCTCCAAC	AA	TTggCCAggggTgCTAAgCAgTTgg
21	CCTCAACCTACCTCCAAC	AA	TCACgCCACAgTTTCCCggAggggC
25	CCTCAACCTACCTCCAAC	AA	CggAAggCCATgCCAgTgAgCTTCC
29	CCTCAACCTACCTCCAAC	AA	AgggggCCCTCCgACgCCTgCTTCA
33	CCTCAACCTACCTCCAAC	AA	TggTCgTTgAgggCAATgCCAgCCC
37	CCTCAACCTACCTCCAAC	AA	CAgggACTCCCCAgCAgTgAgggTC

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
2	TTACCAgAgTTAAAAgCAgCCCTgg	AT	TCTCACCATATTCgCTTC
6	TCAgCCTTgACggTgCCATggAATT	AT	TCTCACCATATTCgCTTC
10	gTggACTCCACgACgTACTCAgCgC	AT	TCTCACCATATTCgCTTC
14	ACACCCATgACgAACATgggggCAT	AT	TCTCACCATATTCgCTTC
18	ACgATACCAAAgTTgTCATggATgA	AT	TCTCACCATATTCgCTTC
22	ATgATgTTCTggAgAgCCCCgCggC	AT	TCTCACCATATTCgCTTC
26	ACCACTgACACgTTggCAgTggggA	AT	TCTCACCATATTCgCTTC
30	TgCTCAgTgTAgCCCAggATgCCCT	AT	TCTCACCATATTCgCTTC
34	TCATACCAggAAATgAgCTTgACAA	AT	TCTCACCATATTCgCTTC
38	TTCAgTgTggTgggggACTgAgTgT	AT	TCTCACCATATTCgCTTC

Organism: ***H. sapiens sapiens***
Target mRNA: **actin beta (*ACTB*)**
Probe set: **10 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B2-Alexa594 (Figure S23)**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
1	CCTCgTAAATCCTCATCA	AA	gCggggCggACgCggTCTCggCggT
5	CCTCgTAAATCCTCATCA	AA	gCTggCggCgggTgTggACgggCgg
9	CCTCgTAAATCCTCATCA	AA	TgCgCAAgTTAggTTTTgTCAAgAA
13	CCTCgTAAATCCTCATCA	AA	ACCAAAACAAAACAAAAAAAACAAA
17	CCTCgTAAATCCTCATCA	AA	CACCTTCACCgTTCCAgTTTTTAAA
21	CCTCgTAAATCCTCATCA	AA	AgTCCTCggCCACATTgTgAACTTT
25	CCTCgTAAATCCTCATCA	AA	ACAACgCATCTCATATTTggAATgA
29	CCTCgTAAATCCTCATCA	AA	ATTCTCCTTAgAgAgAAgTggggTg
33	CCTCgTAAATCCTCATCA	AA	ACACgAAAgCAATgCTATCACCTCC
37	CCTCgTAAATCCTCATCA	AA	CAAAATAAAAAAgTATTAAggCgAA

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
2	gATCggCAAAggCgAggCTCTgTgC	AA	ATCATCCAgTAAACCgCC
6	gCgCggCgATATCATCATCCATggT	AA	ATCATCCAgTAAACCgCC
10	AAgCCATgCCAATCTCATCTTgTTT	AA	ATCATCCAgTAAACCgCC
14	CTgAgTCAAgCCAAAAAAAAAAAAA	AA	ATCATCCAgTAAACCgCC
18	gggATgCTCgCTCCAACCgACTgCT	AA	ATCATCCAgTAAACCgCC
22	ATTAAAAAAACAACAATgTgCAATC	AA	ATCATCCAgTAAACCgCC
26	TTTTAggATggCAAgggACTTCCTg	AA	ATCATCCAgTAAACCgCC
30	TgTgTggACTTgggAgAggACTggg	AA	ATCATCCAgTAAACCgCC
34	TTAAAAAAATTTTgCATTACATAAT	AA	ATCATCCAgTAAACCgCC
38	CACgAAggCTCATCATTCAAAATAA	AA	ATCATCCAgTAAACCgCC

Organism: ***H. sapiens sapiens***
Target mRNA: **phosphoglycerate kinase 1 (*PGK1*)**
Probe set: **18 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B1-Alexa488 (Figures 6B and S25)**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
1	gAggAgggCAgCAAACgg	AA	CCgCCCCTTCCCggCCgCTgCTCTC
5	gAggAgggCAgCAAACgg	AA	CAACgAgggAgCCgACTgCCgACgT
9	gAggAgggCAgCAAACgg	AA	gACTCTCATAACgACCCgCTTCCCT
13	gAggAgggCAgCAAACgg	AA	CTTCCCTTCTTCCTCCACATgAAAg
17	gAggAgggCAgCAAACgg	AA	gCCAAAAgCATCATTgACATAgACA
21	gAggAgggCAgCAAACgg	AA	gCTCTCCAAggCCTTTgCAAAgTAg
25	gAggAgggCAgCAAACgg	AA	CTTCTCAgCTTTggACATTAggTCT
29	gAggAgggCAgCAAACgg	AA	CCAgCCAgCAggTATgCCAgAAgCC
33	gAggAgggCAgCAAACgg	AA	AgCTTCCCATTCAAATACCCCCACA
37	gAggAgggCAgCAAACgg	AA	gTACTAAATATTgCTgAgAgCATCC
41	gAggAgggCAgCAAACgg	AA	ggCCACTAgCTgAATCTTgACATgg
45	gAggAgggCAgCAAACgg	AA	CTAAAAAATTCAAATgggATCTTgA
49	gAggAgggCAgCAAACgg	AA	TAATCATAATAACCTACATCAAAAg
53	gAggAgggCAgCAAACgg	AA	TCAATggACACTTTTATTgTTTACT
57	gAggAgggCAgCAAACgg	AA	CTgCCCCACTTCTTgCATTCAgCAA
61	gAggAgggCAgCAAACgg	AA	CTgATAAAAATAAAAgTTAgAATAA
65	gAggAgggCAgCAAACgg	AA	CAAgAgTTgAAAgTggTCACCTCTg
69	gAggAgggCAgCAAACgg	AA	gAgCCTTCCTCCATggTATgAAATA

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
2	CTACCgCCCCACACCCCgCCTCCCg	TA	gAAgAgTCTTCCTTTACg
6	gCTggggAgAgAggTCggTgATTCg	TA	gAAgAgTCTTCCTTTACg
10	gTTgTTCTTCATAggAACATTgAAg	TA	gAAgAgTCTTCCTTTACg
14	AACCTTgTTCCCAgAAgCATCTTTT	TA	gAAgAgTCTTCCTTTACg
18	CATggAgCTgTgggCTCTgTgAgCA	TA	gAAgAgTCTTCCTTTACg
22	CAggATggCCAggAAgggTCgCTCT	TA	gAAgAgTCTTCCTTTACg
26	AACAggCAAggTAATCTTCACACCA	TA	gAAgAgTCTTCCTTTACg
30	gCTTTCAggACCACAgTCCAAgCCC	TA	gAAgAgTCTTCCTTTACg
34	CATgAgAgCTTTggTTCCCCgggCA	TA	gAAgAgTCTTCCTTTACg
38	TgTgCACAggAACTAAAAggCAggA	TA	gAAgAgTCTTCCTTTACg
42	TTAAgggTTCCTggCACTgCATCTC	TA	gAAgAgTCTTCCTTTACg
46	ACTCTAgAATgCACAATggTTTAgT	TA	gAAgAgTCTTCCTTTACg
50	TgCTgAgTAgTgAAACAgTgACAAA	TA	gAAgAgTCTTCCTTTACg
54	gACAggAAAAAAAAAAAAATCACgg	TA	gAAgAgTCTTCCTTTACg
58	TCTAATTgTCCCATCTCTCCACTgC	TA	gAAgAgTCTTCCTTTACg
62	gACTTTTTAAATTATgATCATgTgT	TA	gAAgAgTCTTCCTTTACg
66	AACATggAggTATATACCTgAAAAA	TA	gAAgAgTCTTCCTTTACg
70	TgAAgAAgTggAAATATATgTggAA	TA	gAAgAgTCTTCCTTTACg

Organism: ***H. sapiens sapiens***
Target mRNA: **phosphoglycerate kinase 1 (*PGK1*)**
Probe set: **18 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B2-Alexa594 (Figures 6B, 6C, S25, and S27)**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
3	CCTCgTAAATCCTCATCA	AA	ACCgCgCgggCAggAACAgggCCCA
7	CCTCgTAAATCCTCATCA	AA	gTTAgAAAgCgACATTTTggAAATA
11	CCTCgTAAATCCTCATCA	AA	CTTAATCCTCTggTTgTTTgTTATC
15	CCTCgTAAATCCTCATCA	AA	ggAgTACTTgTCAggCATgggCACA
19	CCTCgTAAATCCTCATCA	AA	AgCCTTCTgTggCAgATTgACTCCT
23	CCTCgTAAATCCTCATCA	AA	ATCAAACTTgTCAgCAgTgACAAAg
27	CCTCgTAAATCCTCATCA	AA	AgTgACAgCCTCAgCATACTTCTTg
31	CCTCgTAAATCCTCATCA	AA	CCTAgAAgTggCTTTCACCACCTCA
35	CCTCgTAAATCCTCATCA	AA	CTCCAAACTggCACCACCCCCAgTg
39	CCTCgTAAATCCTCATCA	AA	CAgAAAATgCTAAgTTgACTTAggg
43	CCTCgTAAATCCTCATCA	AA	AgATgAgCTgAgATgCTgTgCAACT
47	CCTCgTAAATCCTCATCA	AA	TTTAACAggCAAAATATAAATATAT
51	CCTCgTAAATCCTCATCA	AA	TACAAATggAATTTCATCTTgTTTC
55	CCTCgTAAATCCTCATCA	AA	CTATTCTCACCCTTCCTAACAAAgT
59	CCTCgTAAATCCTCATCA	AA	AggCCCTTgATAAAgAATggACATT
63	CCTCgTAAATCCTCATCA	AA	ggCTggggCTTTTTTgTTATAAgCC
67	CCTCgTAAATCCTCATCA	AA	gTgAACAATATAAgCATATTACTTA
71	CCTCgTAAATCCTCATCA	AA	AATTgTgACAAAACTATACCgAgAg

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
4	gCTCCggAggCTTgCAgAATgCggA	AA	ATCATCCAgTAAACCgCC
8	AACgTCCAgCTTgTCCAgCgTCAgC	AA	ATCATCCAgTAAACCgCC
12	gCAgAATTTgATgCTTgggACAgCA	AA	ATCATCCAgTAAACCgCC
16	TTTgAgTTCTACAgCAACTggCTCT	AA	ATCATCCAgTAAACCgCC
20	CAgCTCCTTCTTCATCAAAAACCCA	AA	ATCATCCAgTAAACCgCC
24	AgTggCTTggCCAgTCTTggCATTC	AA	ATCATCCAgTAAACCgCC
28	ACCATTCCACACAATCTgCTTAgCC	AA	ATCATCCAgTAAACCgCC
32	TCCACCACCTATgATggTgATgCAg	AA	ATCATCCAgTAAACCgCC
36	CCCAggAAggACTTTACCTTCCAgg	AA	ATCATCCAgTAAACCgCC
40	ggTTTTAgCTAATgCCAAgTggAgA	AA	ATCATCCAgTAAACCgCC
44	AATgTATgCAAATCCAgggTgCAgT	AA	ATCATCCAgTAAACCgCC
48	AACTAAgCTAACACTgCTCACTTTC	AA	ATCATCCAgTAAACCgCC
52	ATggATCATCAATTTTgTCTCACTA	AA	ATCATCCAgTAAACCgCC
56	TAgACATCTgATCCgTTCCTCAAgA	AA	ATCATCCAgTAAACCgCC
60	gCACTAgCACAATgTCTgCCATAAA	AA	ATCATCCAgTAAACCgCC
64	gAgTgggAATCTTgAATgggAggAA	AA	ATCATCCAgTAAACCgCC
68	TTTCTTTAAAAAATAAAAAAAAAAg	AA	ATCATCCAgTAAACCgCC
72	gTTATgTAgACTTTTgATCTAATCT	AA	ATCATCCAgTAAACCgCC

Organism: ***E. coli***
Target mRNA: **enhanced green fluorescent protein (*Tg(EGFP)*)**
Probe set: **12 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B3-Alexa594 (Figures 6A and S22)**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
1	gTCCCTgCCTCTATATCT	TT	TgAAAAgTTCTTCTCCTTTACgCAT
3	gTCCCTgCCTCTATATCT	TT	ATTTgTgCCCATTAACATCACCATC
5	gTCCCTgCCTCTATATCT	TT	TAAgggTAAgTTTTCCgTATgTTgC
7	gTCCCTgCCTCTATATCT	TT	TAgTgACAAgTgTTggCCATggAAC
9	gTCCCTgCCTCTATATCT	TT	gCTgTTTCATATgATCTgggTATCT
11	gTCCCTgCCTCTATATCT	TT	ATATAgTTCTTTCCTgTACATAACC
13	gTCCCTgCCTCTATATCT	TT	CACCTTCAAACTTgACTTCAgCACg
15	gTCCCTgCCTCTATATCT	TT	TTCCATCTTCTTTAAAATCAATACC
17	gTCCCTgCCTCTATATCT	TT	TgATgTATACATTgTgTgAgTTATA
19	gTCCCTgCCTCTATATCT	TT	TgTTgTgTCTAATTTTgAAgTTAAC
21	gTCCCTgCCTCTATATCT	TT	CAATTggAgTATTTTgTTgATAATg
23	gTCCCTgCCTCTATATCT	TT	gggCAgATTgTgTggACAggTAATg

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
2	ATTCAACAAgAATTgggACAACTCC	TT	CCACTCAACTTTAACCCg
4	CACCTTCACCCTCTCCACTgACAgA	TT	CCACTCAACTTTAACCCg
6	gTAgTTTTCCAgTAgTgCAAATAAA	TT	CCACTCAACTTTAACCCg
8	CAAAgCATTgAACACCATAACCgAA	TT	CCACTCAACTTTAACCCg
10	CgggCATggCACTCTTgAAAAAgTC	TT	CCACTCAACTTTAACCCg
12	TCTTgTAgTTCCCgTCATCTTTgAA	TT	CCACTCAACTTTAACCCg
14	TTAACTCgATTCTATTAACAAgggT	TT	CCACTCAACTTTAACCCg
16	TgTATTCCAATTTgTgTCCAAgAAT	TT	CCACTCAACTTTAACCCg
18	TgATTCCATTCTTTTgTTTgTCTgC	TT	CCACTCAACTTTAACCCg
20	CTgCTAgTTgAACgCTTCCATCTTC	TT	CCACTCAACTTTAACCCg
22	TgTCTggTAAAAggACAgggCCATC	TT	CCACTCAACTTTAACCCg
24	CTCTCTTTTCgTTgggATCTTTCgA	TT	CCACTCAACTTTAACCCg

Organism: ***E. coli***
Target mRNA: **GTP-binding protein chain elongation factor EF-G *(fusA)***
Probe set: **18 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B2-Alexa594 (Figures 6B and S26)**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
1	CCTCgTAAATCCTCATCA	AA	gTgCgATgggTgTTgTACgAgCCAT
5	CCTCgTAAATCCTCATCA	AA	CATgAACTTCACCgATTTTATggTT
9	CCTCgTAAATCCTCATCA	AA	gCTCATACTgCTTAgCCATACCAgA
13	CCTCgTAAATCCTCATCA	AA	CAACTgCgCAgTAAACCATTACCgC
17	CCTCgTAAATCCTCATCA	AA	TCAggAAgTTCgCACCCATgCggTC
21	CCTCgTAAATCCTCATCA	AA	TCATTTTCACCAggTCAACAACACC
25	CCTCgTAAATCCTCATCA	AA	ATTCgATCAggTTCTggTgCCATTC
29	CCTCgTAAATCCTCATCA	AA	TTTCgTTgTTCAgAACgCgCTgACg
33	CCTCgTAAATCCTCATCA	AA	CgTTgATCgCAggTACgTCAACCgg
37	CCTCgTAAATCCTCATCA	AA	ggTTACCAACAAACgggTCggTAgC
41	CCTCgTAAATCCTCATCA	AA	CgATAgCAgCAgCgATgTCgCCCgC
45	CCTCgTAAATCCTCATCA	AA	TCggTTCAACTgCgATggAgATTAC
49	CCTCgTAAATCCTCATCA	AA	ATTCACgCTTCATACggTCAACgAT
53	CCTCgTAAATCCTCATCA	AA	CACgACCACCAgACTgTTTCgCgTg
57	CCTCgTAAATCCTCATCA	AA	ATTCgCCAgggATTACACCACCTTT
61	CCTCgTAAATCCTCATCA	AA	CATggTAAgAACCgAAgTgCAgACg
65	CCTCgTAAATCCTCATCA	AA	CTTCAACCTTCATgATCggCTCAAg
69	CCTCgTAAATCCTCATCA	AA	CAgCgTggATCTTAACgCCAgTAAC

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
2	gCgCACTgATACCgATgTTACggTA	AA	ATCATCCAgTAAACCgCC
6	CCATCCAgTCCATggTTgCAgCgCC	AA	ATCATCCAgTAAACCgCC
10	gggTgTCgATgATgTTgATgCgATg	AA	ATCATCCAgTAAACCgCC
14	CggTTTCAgACTgCggCTgAACACC	AA	ATCATCCAgTAAACCgCC
18	gACgggTTTTgATCTggTTAACAAC	AA	ATCATCCAgTAAACCgCC
22	ggTCAgCgTCgTTCCAgTTgATAgC	AA	ATCATCCAgTAAACCgCC
26	TCAgCTCTTCAgAAgCTTCAgCTgC	AA	ATCATCCAgTAAACCgCC
30	ACgCAgAACCACAggTTACCAggAT	AA	ATCATCCAgTAAACCgCC
34	gAgTgTCTTTACCgTCgTCCAggAT	AA	ATCATCCAgTAAACCgCC
38	CACCggAgTAAACACggAAgAAggT	AA	ATCATCCAgTAAACCgCC
42	TgTCACCAgTggTTACgTCTTTCAg	AA	ATCATCCAgTAAACCgCC
46	CCATTTTTTCCTggTCAgCTTTggT	AA	ATCATCCAgTAAACCgCC
50	gTTTACCTACgTTCgCTTCAACgTT	AA	ATCATCCAgTAAACCgCC
54	TgTCgATAACAACATgACCATACTg	AA	ATCATCCAgTAAACCgCC
58	ggATACCTTTATCAACggCCgggAT	AA	ATCATCCAgTAAACCgCC
62	TAAACgCCAgTTCAgAggAgTCAAC	AA	ATCATCCAgTAAACCgCC
66	CACCggTgTTCTCTTCCggAgTTTC	AA	ATCATCCAgTAAACCgCC
70	ATCCgAACATTTCAgACAgCggTAC	AA	ATCATCCAgTAAACCgCC

Organism: ***E.coli***
Target mRNA: **GTP-binding protein chain elongation factor EF-G *fusA***
Probe set: **18 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B3-Alexa488 (Figures 6B, S26, and S28)**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
3	gTCCCTgCCTCTATATCT	TT	TAgTAgTggTTTTACCggCgTCgAT
7	gTCCCTgCCTCTATATCT	TT	TggTAATACCACgTTCCTgCTCCTg
11	gTCCCTgCCTCTATATCT	TT	CTTCgATTgTgAAgTCAACgTgCCC
15	gTCCCTgCCTCTATATCT	TT	CTTTATATTTgTTTgCCTgACgCCA
19	gTCCCTgCCTCTATATCT	TT	gCTgCAgCggAACCgggTTCgCgCC
23	gTCCCTgCCTCTATATCT	TT	TATCTTCgTATTCgAAggTTACgCC
27	gTCCCTgCCTCTATATCT	TT	gTTCTTCACCACCCAggTATTTTTC
31	gTCCCTgCCTCTATATCT	TT	gCATCgCCTgAACACCTTTgTTCTT
35	gTCCCTgCCTCTATATCT	TT	CgTCATCACTTgCgTgACgTTCAgC
39	gTCCCTgCCTCTATATCT	TT	TCAgTACggTATCACCAgAgTTAAC
43	gTCCCTgCCTCTATATCT	TT	TgATCggCgCATCCgggTCACACAg
47	gTCCCTgCCTCTATATCT	TT	CTTTAgCCAgACggCCCAgAgCCAg
51	gTCCCTgCCTCTATATCT	TT	ggATAgTTTCACggTAAgCAACCTg
55	gTCCCTgCCTCTATATCT	TT	ggTTTgAACCCggCTCCAgCgggTA
59	gTCCCTgCCTCTATATCT	TT	CCAgCggACCTgCTTTCAgCTgTTC
63	gTCCCTgCCTCTATATCT	TT	CTTTAAAggCgATAgAAgCAgCCAg
67	gTCCCTgCCTCTATATCT	TT	gACgACggCTCAAgTCACCgATAAC
71	gTCCCTgCCTCTATATCT	TT	TggTCAgAgAACgCAgCTgAgTTgC

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
4	CACCggTgTAgAACAgAATACgTTC	TT	CCACTCAACTTTAACCCg
8	AgAATgCAgTAgTCgCAgCggAAgT	TT	CCACTCAACTTTAACCCg
12	CATCgAgAACACgCATggAACgTTC	TT	CCACTCAACTTTAACCCg
16	TTTTgTTAACgAACgCAATgCgCgg	TT	CCACTCAACTTTAACCCg
20	TgAAATgTTCTTCAgCACCAATCgC	TT	CCACTCAACTTTAACCCg
24	TAgCCAgTTCAACCATgTCTgCCgg	TT	CCACTCAACTTTAACCCg
28	gAgCACCTTTgATTTCTgCTTCAgT	TT	CCACTCAACTTTAACCCg
32	ATggCAggTAATCAATTACCgCATC	TT	CCACTCAACTTTAACCCg
36	TTTTgAACgCCAgTgCAgAgAACgg	TT	CCACTCAACTTTAACCCg
40	AACgCTCACgTgCAgCTTTCACggA	TT	CCACTCAACTTTAACCCg
44	gCTCAgggAATTCCATACgTTCCAg	TT	CCACTCAACTTTAACCCg
48	CAgTCCATACACggAAAgACgggTC	TT	CCACTCAACTTTAACCCg
52	TACCTTCAACATCggTAACTTTCTg	TT	CCACTCAACTTTAACCCg
56	TgTCgTTgATgAACTCgTAgCCTTT	TT	CCACTCAACTTTAACCCg
60	TACCCATgTCTACTACCgggTAgCC	TT	CCACTCAACTTTAACCCg
64	gAACTggTTTCgCTTTCTTAAAgCC	TT	CCACTCAACTTTAACCCg
68	CAgATTCCTgACCTTTgAgCATACC	TT	CCACTCAACTTTAACCCg
72	ATTCCATAgTgTATgATgCACgACC	TT	CCACTCAACTTTAACCCg

Organism: ***E. coli***
Target mRNA: **isocitrate dehydrogenase (*icd*)**
Probe set: **20 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B1-Alexa594 (Figure S28)**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
1	gAggAgggCAgCAAACgg	AA	CCggAACAACTACTTTACTTTCCAT
3	gAggAgggCAgCAAACgg	AA	gATTTTCAggAACgTTgAgTTTgCC
5	gAggAgggCAgCAAACgg	AA	TggCTggggTTACATCTACACCgAT
7	gAggAgggCAgCAAACgg	AA	TTTTACgCTCgCCTTTATAggCTTT
9	gAggAgggCAgCAAACgg	AA	ACCTgACCATAAACCTgTgTggATTT
11	gAggAgggCAgCAAACgg	AA	TggCAACgCgATATTCACgAATCAg
13	gAggAgggCAgCAAACgg	AA	CAACgTTCAgAgAgCgAATACCgCC
15	gAggAgggCAgCAAACgg	AA	gATAgTAACgTACCggACgCAggCA
17	gAggAgggCAgCAAACgg	AA	ggAAgATAACCATATCggTCAgTTC
19	gAggAgggCAgCAAACgg	AA	CggCAgAgTCTgCTTTCCATTCgAT
21	gAggAgggCAgCAAACgg	AA	gAATTTTCTTCACCCCCATCTCTTC
23	gAggAgggCAgCAAACgg	AA	TggTgCCTTCTTCCgAACACggCTT
25	gAggAgggCAgCAAACgg	AA	CAgAgTCACgATCgTTAgCAATTgC
27	gAggAgggCAgCAAACgg	AA	CTTTAAACgCTCCTTCggTgAACTT
29	gAggAgggCAgCAAACgg	AA	CACCgTCgATCAgTTCACCgCCAAA
31	gAggAgggCAgCAAACgg	AA	CTTTAATgACgATCTCTTTgCCAgT
33	gAggAgggCAgCAAACgg	AA	CATATTCAgCCggACgCAgCAggAT
35	gAggAgggCAgCAAACgg	AA	CTgCCAgggCgTCAgAAATgTAgTC
37	gAggAgggCAgCAAACgg	AA	CgCATTCgTCACCgATgTTTgCACC
39	gAggAgggCAgCAAACgg	AA	CTTTgTCCTgACCggCATATTTCgg

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
2	TTTgCAgggTgATCTTCTTgCCTTg	TA	gAAgAgTCTTCCTTTACg
4	CATCACCTTCAATgTAAgggATAAT	TA	gAAgAgTCTTCCTTTACg
6	CgACTgCAgCgTCgACCACTTTCAg	TA	gAAgAgTCTTCCTTTACg
8	CACCggTgTAAATTTCCATCCAggA	TA	gAAgAgTCTTCCTTTACg
10	CAAgAgTTTCAgCAggCAgCCAgAC	TA	gAAgAgTCTTCCTTTACg
12	CAACCggAgTggTCAgCggACCTTT	TA	gAAgAgTCTTCCTTTACg
14	TgTAgAgATCCAgTTCCTggCgCAg	TA	gAAgAgTCTTCCTTTACg
16	ggTgTTTAACCgggCTTggAgTgCC	TA	gAAgAgTCTTCCTTTACg
18	CCgCATAAATgTCTTCCgAgTTTTC	TA	gAAgAgTCTTCCTTTACg
20	gCAggAATTTAATCACTTTCTCggC	TA	gAAgAgTCTTCCTTTACg
22	TACCgATACCACAATgTTCCgggAA	TA	gAAgAgTCTTCCTTTACg
24	ATTCgATCgCTgCACgAACCAgACg	TA	gAAgAgTCTTCCTTTACg
26	TgATgTTgCCTTTgTgCACCAgAgT	TA	gAAgAgTCTTCCTTTACg
28	CTTCACgCgCCAgCTggTAgCCCCA	TA	gAAgAgTCTTCCTTTACg
30	TCgggTTTTTAACTTTCAgCCACgg	TA	gAAgAgTCTTCCTTTACg
32	gTTgCAggAATgCATCAgCAATCAC	TA	gAAgAgTCTTCCTTTACg
34	CgTTCAggTTCATACAggCgATAAC	TA	gAAgAgTCTTCCTTTACg
36	gggCgATACCgATACCgCCAACCTg	TA	gAAgAgTCTTCCTTTACg
38	CAgTACCgTgggTggCTTCAAACAg	TA	gAAgAgTCTTCCTTTACg
40	CggAgAgAATAATAgAgCCAggATT	TA	gAAgAgTCTTCCTTTACg

S4.9 Split-initiator probes for Figures 7, S29, and S30

Organism: ***H. sapiens sapiens***
Target mRNA: **B-Raf proto-oncogene, serine/threonine kinase (*BRAF*)**
Probe set: **23 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B3-Alexa647**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
1	gTCCCTgCCTCTATATCT	TT	ACCgCTCAgCgCCgCCATCTTATAA
5	gTCCCTgCCTCTATATCT	TT	ggCAgggTCCgCAgCCgAAgAggCC
9	gTCCCTgCCTCTATATCT	TT	ATATATTgATggTggATTATgCTCC
13	gTCCCTgCCTCTATATCT	TT	AgAgCTAgAAACAgAAAAATCAgTT
17	gTCCCTgCCTCTATATCT	TT	CTTggggTTgCTCCgTgCCACATCT
21	gTCCCTgCCTCTATATCT	TT	CTCTCCATCCTgAATTCTgTAAACA
25	gTCCCTgCCTCTATATCT	TT	TTTTCgTACAAAgTTgTgTgTTgTA
29	gTCCCTgCCTCTATATCT	TT	AACTTCTgTACTACAACgCTggTgA
33	gTCCCTgCCTCTATATCT	TT	TAgggCAgTCTCTgCTAAggACgCC
37	gTCCCTgCCTCTATATCT	TT	TggTCggAAgggCTgTggAATTggA
41	gTCCCTgCCTCTATATCT	TT	TCTAATCAAgTCATCAATATTgACA
45	gTCCCTgCCTCTATATCT	TT	TggAgATTTCTgTAAggCTTTCACg
49	gTCCCTgCCTCTATATCT	TT	CTgCCCATCAggAATCTCCCAATCA
53	gTCCCTgCCTCTATATCT	TT	AggTgTAggTgCTgTCACATTCAAC
57	gTCCCTgCCTCTATATCT	TT	ggTCTCAATgATATggAgATggTgA
61	gTCCCTgCCTCTATATCT	TT	CACTgTAgCTAgACCAAAATCACCT
65	gTCCCTgCCTCTATATCT	TT	CTgAAAgCTgTATggATTTTTATCT
69	gTCCCTgCCTCTATATCT	TT	ACTAAAATCCTCTgTTTggAAACCA
73	gTCCCTgCCTCTATATCT	TT	TTTTgTTgCTACTCTCCTgAACTCT
77	gTCCCTgCCTCTATATCT	TT	ATTATATCTAgTCTTTAACCACACA
81	gTCCCTgCCTCTATATCT	TT	AAgTAAAgCCTCTAgAAgAggCTCT
85	gTCCCTgCCTCTATATCT	TT	TCTTCTggAgTCCCTAgTggACATg
89	gTCCCTgCCTCTATATCT	TT	ATTAAATTCTACTgACTTCCTAAAT

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
2	gCCCggCTCCgCgCCgCCACCACCg	TT	CCACTCAACTTTAACCCg
6	TTTgATATTCCACACCTCCTCCggA	TT	CCACTCAACTTTAACCCg
10	gCTggTgTATTCTTCATAggCCTCC	TT	CCACTCAACTTTAACCCg
14	AgAAgATgTAACggTATCCATTgAT	TT	CCACTCAACTTTAACCCg
18	gACTCTAACgATAggTTTTTgTggT	TT	CCACTCAACTTTAACCCg
22	ATCAgTgTCCCAACCAATTggTTTC	TT	CCACTCAACTTTAACCCg
26	gTCACAAAATgCTAAggTgAAAAAC	TT	CCACTCAACTTTAACCCg
30	TTggTCATAATTAACACACATCAgT	TT	CCACTCAACTTTAACCCg
34	gggTgCggAAggggATgATCCAgAT	TT	CCACTCAACTTTAACCCg
38	AAATTgATTTCgATgATCTTCATCT	TT	CCACTCAACTTTAACCCg
42	TCCTCCATCACCACgAAATCCTTgg	TT	CCACTCAACTTTAACCCg
46	AgATgACTTCCTTTCTCgCTgAggT	TT	CCACTCAACTTTAACCCg
50	AgATCCAATTCTTTgTCCCACTgTA	TT	CCACTCAACTTTAACCCg
54	TTCATTTTTgAAggCTTgTAACTgC	TT	CCACTCAACTTTAACCCg
58	ATCTATAAgTTTgATCATCTCAAAT	TT	CCACTCAACTTTAACCCg
62	CTgATgggACCCACTCCATCgAgAT	TT	CCACTCAACTTTAACCCg
66	AACAATTCCAAATgCATATACATCT	TT	CCACTCAACTTTAACCCg
70	TgTTTTTggAgAAgCACAAgCATAT	TT	CCACTCAACTTTAACCCg
74	AAgCAAACATATgTTCATTTATTTT	TT	CCACTCAACTTTAACCCg
78	TAAgTATAAATTTTAgTTTggggAA	TT	CCACTCAACTTTAACCCg
82	AAgTgAATgATACAAACCCggAACA	TT	CCACTCAACTTTAACCCg
86	CTgCAAACACAggCATAggTAgggT	TT	CCACTCAACTTTAACCCg
90	AAAATTATTAAgAATAATAATAgAA	TT	CCACTCAACTTTAACCCg

Organism: ***H. sapiens sapiens***
Target mRNA: **B-Raf proto-oncogene, serine/threonine kinase (*BRAF*)**
Probe set: **23 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B4-Alexa546**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
3	CCTCAACCTACCTCCAAC	AA	CTCCATgTCCCCgTTgAACAgAgCC
7	CCTCAACCTACCTCCAAC	AA	ATgTTCCTgTgTCAACTTAATCATT
11	CCTCAACCTACCTCCAAC	AA	TTCTCTTTgTTggAgTgCATCTAgC
15	CCTCAACCTACCTCCAAC	AA	AggTAgCACTgAAAggCTAgAAgAg
19	CCTCAACCTACCTCCAAC	AA	CACTgTCCTCTgTTTgTTgggCAgg
23	CCTCAACCTACCTCCAAC	AA	CAATTCTTCTCCAgTAAgCCAggAA
27	CCTCAACCTACCTCCAAC	AA	ACCCTggAAAAgCAgCTTTCgACAA
31	CCTCAACCTACCTCCAAC	AA	gAACTTggAgACAAACAgCAAATCA
35	CCTCAACCTACCTCCAAC	AA	AATTTggggCCCAATAgAgTCCgAg
39	CCTCAACCTACCTCCAAC	AA	AgCTgATgAggATCggTCTCgTTgC
43	CCTCAACCTACCTCCAAC	AA	gggggTAgCAgACAAACCTgTggTT
47	CCTCAACCTACCTCCAAC	AA	CATTCgATTCCTgTCTTCTgAggAT
51	CCTCAACCTACCTCCAAC	AA	TCCCTTgTAgACTgTTCCAAATgAT
55	CCTCAACCTACCTCCAAC	AA	ATgTCgTgTTTTCCTgAgTACTCCT
59	CCTCAACCTACCTCCAAC	AA	TATATTATTACTCTTgAggTCTCTg
63	CCTCAACCTACCTCCAAC	AA	CAAAATggATCCAgACAACTgTTCA
67	CCTCAACCTACCTCCAAC	AA	CTTCATggCTTTTggACAgTTACTC
71	CCTCAACCTACCTCCAAC	AA	CgCACCATATCCCCCTgCCTggATg
75	CCTCAACCTACCTCCAAC	AA	gAgAgTATTTTATTCAATTTAACAT
79	CCTCAACCTACCTCCAAC	AA	AACCCTTggATgTTAAAAATCCAAT
83	CCTCAACCTACCTCCAAC	AA	AACTgAAgTTTACTACTTAAAATAA
87	CCTCAACCTACCTCCAAC	AA	CAggCTAACCgACTgCCAACTTCTC
91	CCTCAACCTACCTCCAAC	AA	ATTgTTATAAAAAgAAATAgTTATA

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
4	ggCgCCggCgCCggCgCCggCCTCg	AT	TCTCACCATATTCgCTTC
8	ACCAAATTTgTCCAATAgggCCTCT	AT	TCTCACCATATTCgCTTC
12	gTTCCCCAgAgATTCCAATAACTgT	AT	TCTCACCATATTCgCTTC
16	gggATTTTgAAAAACTgAAAgAgAT	AT	TCTCACCATATTCgCTTC
20	gACTgTAACTCCACACCTTgCAggT	AT	TCTCACCATATTCgCTTC
24	TggAACATTCTCCAACACTTCCACA	AT	TCTCACCATATTCgCTTC
28	TTTATAACCACATgTTTgACAgCgg	AT	TCTCACCATATTCgCTTC
32	TTCCTgTggTATTgggTggTgTTCA	AT	TCTCACCATATTCgCTTC
36	ggATTTTgAAggAgACggACTggTg	AT	TCTCACCATATTCgCTTC
40	TTCTATTgTgTTTATATgCACATTg	AT	TCTCACCATATTCgCTTC
44	AgTTAgTgAgCCAggTAATgAggCA	AT	TCTCACCATATTCgCTTC
48	ACTCgAgTCCCgTCTACCAAgTgTT	AT	TCTCACCATATTCgCTTC
52	TTTCACTgCCACATCACCATgCCAC	AT	TCTCACCATATTCgCTTC
56	ATAgCCCATgAAgAgTAggATATTC	AT	TCTCACCATATTCgCTTC
60	TTTTACTgTgAggTCTTCATgAAgA	AT	TCTCACCATATTCgCTTC
64	CATTCTgATgACTTCTggTgCCATC	AT	TCTCACCATATTCgCTTC
68	CTTTTTgAggCACTCTgCCATTAAT	AT	TCTCACCATATTCgCTTC
72	CACTCATTTgTTTCAgTggACAggA	AT	TCTCACCATATTCgCTTC
76	TgTTCTTTggTTCACCTTAAAAAAA	AT	TCTCACCATATTCgCTTC
80	CAATTTTTAgCAATgTCTATgTATT	AT	TCTCACCATATTCgCTTC
84	ATAgCTggCAACAAAAgTTgCATgA	AT	TCTCACCATATTCgCTTC
88	gATCTgTTCAgTTTgCCTTATCTAA	AT	TCTCACCATATTCgCTTC
92	gAAATAAAAgACATCCACATTTTCC	AT	TCTCACCATATTCgCTTC

Organism: ***G. gallus domesticus***
Target mRNA: **diencephalon/mesencephalon homeobox 1 (*Dmbx1*)**
Probe set: **25 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B1-Alexa594**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
1	gAggAgggCAgCAAACgg	AA	CgCTCAgTgAgTTCATggCATgCAg
5	gAggAgggCAgCAAACgg	AA	AAATgATgTCAgCCAgTCTCTCCgC
9	gAggAgggCAgCAAACgg	AA	AAATgATgTCAgCCAgTCTCTCCgC
13	gAggAgggCAgCAAACgg	AA	CAgTgTCAggTATTgTggACTgggT
17	gAggAgggCAgCAAACgg	AA	CCTCCTCTCTgTCAgTCTggTCCTC
21	gAggAgggCAgCAAACgg	AA	CgCTgATgggAgACTCTgATTTTgg
25	gAggAgggCAgCAAACgg	AA	CTgCCATgTgCTggCggAATTgCTC
29	gAggAgggCAgCAAACgg	AA	gACAgTgCAgggAgCTCAgAggCgC
33	gAggAgggCAgCAAACgg	AA	TCTCAATACTTgTTgTTTTACTgTT
37	gAggAgggCAgCAAACgg	AA	gACCTCCggTgCATCTTCTTATggg
41	gAggAgggCAgCAAACgg	AA	CggTgCTAgTAAgACATTAgTAAAT
45	gAggAgggCAgCAAACgg	AA	TCACAgCAgTCCAAAgggACAgTTC
49	gAggAgggCAgCAAACgg	AA	gTTTTCCCAAAgAATgCATCgACAA
53	gAggAgggCAgCAAACgg	AA	ggggAATAAAAgCAAAAgAggCCAC
57	gAggAgggCAgCAAACgg	AA	TTTgCTCTAAgCACCATTAAgACTC
61	gAggAgggCAgCAAACgg	AA	ggAAgTgCTTAAACAggAAATTCAC
65	gAggAgggCAgCAAACgg	AA	AATTTggCTTTCATTTTTCTCCCCA
69	gAggAgggCAgCAAACgg	AA	CTAgACCAAAATgCTCTCCAAAAAg
73	gAggAgggCAgCAAACgg	AA	TAAgAACAgCTTgCATTAATCgTgg
77	gAggAgggCAgCAAACgg	AA	CTTggCCTCCAgCATTgCAgCATTT
81	gAggAgggCAgCAAACgg	AA	gAAATCACTTTgCAgTTggTgAgTT
85	gAggAgggCAgCAAACgg	AA	AAgTCCTTTgggTTggTAgAAAAgT
89	gAggAgggCAgCAAACgg	AA	gCATTTTTggTCCTAggCAATACTA
93	gAggAgggCAgCAAACgg	AA	gACCAgATgATggCCTgCAgTgAAT
97	gAggAgggCAgCAAACgg	AA	AggTAACTCAACCCACggCTTCTgC

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
2	CTgCCTgCTggTgCAggTTgTACAT	TA	gAAgAgTCTTCCTTTACg
6	ggTgCTgggATCCATAACgTgCCTC	TA	gAAgAgTCTTCCTTTACg
10	gCTTCTgCAgCTgCTCCTTCTggAg	TA	gAAgAgTCTTCCTTTACg
14	CCTCACTTgggATgCTCTgAgTCTT	TA	gAAgAgTCTTCCTTTACg
18	TAgCCTCATCCAAggTgCTCTTAAA	TA	gAAgAgTCTTCCTTTACg
22	CACTgCTAgATgAAggAgTCACggT	TA	gAAgAgTCTTCCTTTACg
26	AggAgTAATggACCAAgTTgTTggT	TA	gAAgAgTCTTCCTTTACg
30	CgTgggAgAgAgATTggTAgTACgA	TA	gAAgAgTCTTCCTTTACg
34	CATgCTgCTTTgCCCggAgCCTTAg	TA	gAAgAgTCTTCCTTTACg
38	ggTTCCAggAgTgACATgTCTggTg	TA	gAAgAgTCTTCCTTTACg
42	AgCCAgTAgCAgTgTCTgATgCAAT	TA	gAAgAgTCTTCCTTTACg
46	gCTCTTTCTgAATgTTACAggCTTA	TA	gAAgAgTCTTCCTTTACg
50	TATgTACAAgACAAAgCAggACTCT	TA	gAAgAgTCTTCCTTTACg
54	gACTAgCTACCAAAACTgAgAgAgA	TA	gAAgAgTCTTCCTTTACg
58	gAgCAgTgAATTgCATAATggTTTT	TA	gAAgAgTCTTCCTTTACg
62	AgTAAAggAAAAAACACTTgCCTTT	TA	gAAgAgTCTTCCTTTACg
66	AACAATCAAgTCAAAAgTAACCATg	TA	gAAgAgTCTTCCTTTACg
70	AgTTTTTATTgTTCTCTATTgTCgA	TA	gAAgAgTCTTCCTTTACg
74	TAgAATTTggTgATCggAgCgTTTT	TA	gAAgAgTCTTCCTTTACg
78	AATAgAAAgCCCCCgATTATCACCC	TA	gAAgAgTCTTCCTTTACg
82	AgAgAAATgAggCCAAAACTgTggA	TA	gAAgAgTCTTCCTTTACg
86	TATTTggTTTggAgAAgATAAATAA	TA	gAAgAgTCTTCCTTTACg
90	ACCTAgCACCTgCCACAgAgCCAgT	TA	gAAgAgTCTTCCTTTACg
94	CTCCATTTCTTCTTTAAATCgAgCA	TA	gAAgAgTCTTCCTTTACg
98	AACACCCTTTCCCCCTCgTgTTTAA	TA	gAAgAgTCTTCCTTTACg

Organism: ***G. gallus domesticus***
Target mRNA: **diencephalon/mesencephalon homeobox 1 (*Dmbx1*)**
Probe set: **25 split-initiator probe pairs (each probe carries half an HCR initiator)**
HCR amplifier: **B2-Alexa647**

Odd #	1st Half of Initiator I1	Spacer	Probe Sequence (25 nt)
3	CCTCgTAAATCCTCATCA	AA	AgTCgggCgCgTgCTgTgCTTgCTg
7	CCTCgTAAATCCTCATCA	AA	AggCCgTgCgACTTCgCCTTTgTTT
11	CCTCgTAAATCCTCATCA	AA	CACTgTggCTgCTTTCACAgTCCTT
15	CCTCgTAAATCCTCATCA	AA	ACAgggTTAggTTgAggTCTgTgCT
19	CCTCgTAAATCCTCATCA	AA	CgTCCACACCAggACTCTTgTCCAC
23	CCTCgTAAATCCTCATCA	AA	AggAgTAggAgTgAgTTTgAgCCAg
27	CCTCgTAAATCCTCATCA	AA	TggCAgACggTgTCCCCATTTCgAA
31	CCTCgTAAATCCTCATCA	AA	ggATgggTgAggACCAgACCTgCTg
35	CCTCgTAAATCCTCATCA	AA	gTAgggTgTCAAgCCCAAgggATgC
39	CCTCgTAAATCCTCATCA	AA	ACTCCAggAAgAgATgAgggTggAA
43	CCTCgTAAATCCTCATCA	AA	TCCTCCTCAAATATTTAAAgAAgAC
47	CCTCgTAAATCCTCATCA	AA	ACATTATCgCAgggATgAggTgAgg
51	CCTCgTAAATCCTCATCA	AA	gCggTggATgCTTTCAACATTgTAA
55	CCTCgTAAATCCTCATCA	AA	gggAgCgTggCTgATTTgTgACTTT
59	CCTCgTAAATCCTCATCA	AA	TCAgCTTTAgCAgAgAAgAgAgAAg
63	CCTCgTAAATCCTCATCA	AA	TCTgTCTgTgAACAAgTgCTATTAg
67	CCTCgTAAATCCTCATCA	AA	TTACACTTCACTgAAgACCAAAgAg
71	CCTCgTAAATCCTCATCA	AA	gTATgAACACAgTgggAgTTCATAC
75	CCTCgTAAATCCTCATCA	AA	AgggTCTgAAgCTgCACAgCTTgAg
79	CCTCgTAAATCCTCATCA	AA	CAAgCCAATCTACTCCTCgCTgCAg
83	CCTCgTAAATCCTCATCA	AA	TggATTACTAAAATgAAgggTCATT
87	CCTCgTAAATCCTCATCA	AA	TTCTTggTACggTgAgTTCAAAggA
91	CCTCgTAAATCCTCATCA	AA	gACCCgCCTgACACCCTTTggATTC
95	CCTCgTAAATCCTCATCA	AA	gTTATgTAggCTATgCACACgTTgC
99	CCTCgTAAATCCTCATCA	AA	TTTTTAAgATgCATTATTgCAgTTg

Even #	Probe Sequence (25 nt)	Spacer	2nd Half of Initiator I1
4	gggTgAgggCgTgCACTgAgggCCg	AA	ATCATCCAgTAAACCgCC
8	CCAgTgCCTCCAgCTgTTgggCAgT	AA	ATCATCCAgTAAACCgCC
12	CCAgCACAggTggTTCCgTCTTCCC	AA	ATCATCCAgTAAACCgCC
16	gCgCTgATTCACTggCTgACTgCTC	AA	ATCATCCAgTAAACCgCC
20	TCgCTCTCTTgCAgTTCAAAgCCTT	AA	ATCATCCAgTAAACCgCC
24	gCAggCggAAgAggCTCAggggTgA	AA	ATCATCCAgTAAACCgCC
28	TgTTgACATTCATgCCCAAgTAggg	AA	ATCATCCAgTAAACCgCC
32	ggCTTggAAgAgAgCTggAggCCTg	AA	ATCATCCAgTAAACCgCC
36	ACggTTgCCCATCTggCAgTCAgTT	AA	ATCATCCAgTAAACCgCC
40	AAAgTTTTCCCTgATAgggAgCACC	AA	ATCATCCAgTAAACCgCC
44	CTgTCTAAACACACATCCTCTCCCT	AA	ATCATCCAgTAAACCgCC
48	AAAAAgggTgTATATAACACggTTg	AA	ATCATCCAgTAAACCgCC
52	TTCTgTAACACTgACAgTAACACAC	AA	ATCATCCAgTAAACCgCC
56	AACCCAAgAAgAgCAACTAgCTgTg	AA	ATCATCCAgTAAACCgCC
60	TTgCATCATTTCCTggCCgTTATAA	AA	ATCATCCAgTAAACCgCC
64	CAgCAgCATTTggCCAgCATTTTgT	AA	ATCATCCAgTAAACCgCC
68	AACCCCATAATTTgTAAATgggggA	AA	ATCATCCAgTAAACCgCC
72	TTgTCAAggAACCATATAATTCATg	AA	ATCATCCAgTAAACCgCC
76	CACTTgTTACATTCTCACTTgCTAA	AA	ATCATCCAgTAAACCgCC
80	ggTTgCTTggggACATggTACTTTT	AA	ATCATCCAgTAAACCgCC
84	CTTCTCAAgAAggAAAAACACTCTg	AA	ATCATCCAgTAAACCgCC
88	TTAgATCTgggTTTCCTCCCTCCCT	AA	ATCATCCAgTAAACCgCC
92	gCCCAgCTCTgCTgCgTgTTAgTgg	AA	ATCATCCAgTAAACCgCC
96	TggTATgAAgTAAgATgggAgCAAg	AA	ATCATCCAgTAAACCgCC
100	TgCCTCAgTTTAAgggATTTAgATg	AA	ATCATCCAgTAAACCgCC

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