Chapter 5

Sensitive multiplexed northern blots via hybridization chain reaction (HCR)

5.1 Introduction

In Chapter 2, we presented a mechanism for conditional RNAi activation based on scRNAs. Despite being able to design a system that is functional *in vitro*, the mechanism did not lead to gene silencing in tissue culture. One concern was the degradation of scRNAs in cells. In Chapter 3, we examined the fate of transfected or expressed scRNAs using northern hybridization. Since the mechanism is comprised of three different scRNAs, three separate northern blots are needed (one per scRNA). For expressed scRNAs, when no signal was detected on the blot, a concern we had was whether the scRNAs are being expressed, or whether the expression level is too low to be detected. Detection of low expression levels might also be a concern for conditional shRNA transcription presented in Chapter 4; this has not been studied. To solve these issues, our aim was to develop a sensitive multiplexed northern blot hybridization assay. For gene expression studies, a sensitive multiplexed assay would allow blotting a control reference gene as well as multiple genes of interest in one single experiment.

5.1.1 Northern blot

Northern blot is a hybridization-based technique which was developed as a variation of an older method used to detect DNA, the Southern blot [1]. The purpose of this method is to identify the presence and size of a specific RNA within a sample. The northern blot protocol includes the following main steps: RNA isolation, electrophoretic separation of RNA under denaturing conditions, RNA transfer and crosslinking to a membrane, hybridization of the membrane with a probe for the gene of interest and visualization of signal [2, 3]. The location of the signal obtained by the probe indicates the size of the RNA while the intensity of the signal provides information about the quantity of bound RNA.

Northern blot is not typically used for quantitative analysis and is considered to be semi-quantitative, providing information regarding relative RNA expression within a sample or across samples [3, 4]. To compare RNA between different samples, at least two probes must be used. The first probe targets the gene of interest and the second probe is used as a normalizing control, targeting a second gene that should be present in equal amounts across all samples. Though it is possible to hybridize the sample and the control probe concurrently [5] (if the two targets have distinct size), this is not commonly practiced. Instead, after hybridization and visualization of the first probe, the membrane is re-stripped of the bound probe and hybridized again to detect a second target (e.g., the control). Stripping and re-probing of the membrane can be difficult and reduce sensitivity [2].

5.1.2 Northern blot probes and detection strategies

Northern blot is a versatile technique; the length, base chemistry, and detection moiety can all be changed [2]. Short oligonucleotide probes [6, 7] can be used as well as full- [5] or partial-length cDNA [8]. Because hybridization is based on base pairing, alternative bases to DNA and RNA can be used, depending on probe length. For example, short probes can be made of 2′-OMe, locked nucleic acids (LNA) [9, 10], etc. For detection, both radioactive and non-radioactive probes can be used. Labeling strategies include end-

labeling [11, 12] as well as uniform probe labeling. Uniform labeling can be achieved using random-primer [13], nick translation [14, 15] and transcription reactions [16–18], all of which are rapid methods that provide high-density labeling. Due to its high sensitivity, radioactive labeling with ^{32}P is most commonly used. However, the health hazard, safety measurements and special training associated with radiation use as well as short half-life of the probe are a great disadvantage. Non-isotopic probes include the use of fluorescently labeled nucleotides or haptens (e.g digoxigenin or biotin) [19–22] which are incorporated into the probe. While fluorescent probes can be visualized directly, haptens are detected by an antibody conjugated to alkaline phosphatase (AP) or horseradish peroxidase (HRP) via chemiluminescent detection [6, 8, 23]. Similarly, both AP and HRP can be directly conjugated to the probe and assayed by chemiluminescence [21].

5.1.3 Northern blot in comparison to other methods

While northern blot is widely used throughout the field, new techniques offer advantages over it. Mainly, most newer techniques are considered to be more sensitive, and they also tend to be less sensitive to RNA degradation. Real-time PCR, nuclease protection assays, and fluorescent *in situ* hybridization (FISH) allow examination of multiple genes at once, and microarrays are high-throughput. FISH does not require the isolation of RNA, and in addition, provides information about RNA localization within the cell or tissue. However, no single method provides comprehensive information and so a thorough analysis of gene expression often requires the use of multiple techniques. The only method that provides information regarding both sequence and length is the northern blot. For this reason, northern blot is still widely used for RNA detection and to validate results obtained with other methods. It also provides information about the RNA condition (e.g., degradation) and it can distinguish between splice variants [2, 3].

As previously mentioned, the need to strip and re-probe a northern blot membrane in order to detect multiple targets is a limitation of the technique. To the best of our

knowledge, only one attempt has been made to develop a multiplexed northern blot assay. Hoeltke et al. [24] have developed a colorimetric multiplexed northern blot protocol. Differentially labeled probes have been used against two different targets. Probe labeling was done with digoxigenin or biotin. Each label is detected by alkaline phosphatase conjugate and three different naphtol-AS^3-phosphate/diazonium salt combinations as substrates to AP. While hybridization to the different probes can be carried out simultaneously, label detection is carried out sequentially. Between each detection step, a heat/EDTA treatment is carried out to inactivate the formerly bound AP. The results are visualized as blue and red bands; if the target is bound by more than one probe, the signal results in a mixed color. The great advantage of this method is that it removes the need to strip and re-probe a membrane for detection of more than one target. However, it is still necessary to perform the detection reaction consecutively. Unfortunately, the dyes used in order to detect multiple colors at once are less sensitive than other colorimetric detection dyes; no other dye combination with improved sensitivity has been reported, thus making this method less applicable. Fluorescently labeled northern blot probes can be generated, however, they are considered to be less sensitive than other detection methods and are therefore not widely used [21].

In this chapter, we explore the use of a hybridization chain reaction (HCR) in order to simultaneously detect multiple targets on a northern blot.

5.1.4 Hybridization chain reaction (HCR)

In an HCR system [25], two complementary hairpins are designed to be kinetically trapped in a monomer state. Each hairpin has two single-stranded regions: a toehold and a loop. The complement to the toehold of one hairpin is sequestered in the loop of the other hairpin (and vice versa), kinetically trapping the hairpins. Upon the presence of an initiator, a cascade of reactions begins in which the hairpins form a nicked double-stranded polymer. In Figure 5.1(a) hairpins H1 and H2 are kinetically trapped. Upon the presence of initiator

I in Figure 5.1(b) the toehold 'a' of hairpin H1 base-pairs with its complementary target 'a*' in initiator I. Next, the stem 'b' of hairpin H1 binds to 'b*' in the initiator, the complex I·H1 is formed. Following the binding of H1 to the initiator I the loop 'c' and half of the stem 'b*' of hairpin H1 are exposed as single-stranded regions. In the next step (Figure 5.1(c)), toehold 'c*' of hairpin H2 binds to 'c' in the complex I·H1, followed by binding of the stems 'b-b*' and in the formation of a complex I·H1·H2. Once hairpin H2 is bound and open, a single-stranded region 'a* -b*' is exposed. This region is identical to the original initiator sequence and thus a new H1 hairpin can add to the polymer followed by another H2 hairpin, leading to the formation of a long alternating H1·H2 polymer.

Our lab has recently utilized HCR to develop a multiplexed fluorescent *in situ* hybridization method [26]. Gene probes were designed to include a single-stranded "tail" region which is an HCR initiator sequence. Orthogonal HCR polymers were designed, each initiated by a different initiator sequence. The HCR polymers are made fluorescent by attaching fluorescent dyes to the hairpin monomers. Multiple fluorescent polymers can be grown off the same mRNA by using multiple probes (with the same initiator sequence) against a target gene. Using this method, five mRNAs were detected in a single experiment within a single biological sample. In order to detect other mRNAs, all that is needed is to design a new probe complementary to a gene of interest.

In this chapter, HCR is utilized to improve the northern blot method. A great disadvantage of a northern blot is that in order to detect multiple genes, current practice requires stripping and re-probing the blot. This is time consuming, difficult and may reduce sensitivity of the blot. By using multiplexed fluorescent HCR as a detection moiety of probe hybridization, we can detect four targets simultaneously; this is demonstrated for both mRNA targets and miRNA targets. Fluorescence detection is not as sensitive as the standard radio-isotope ^{32}P. By using a radioactive HCR polymer, we demonstrate miRNA detection of 0.01 femtomol, a 5-fold improvement relative to the current literature [10].

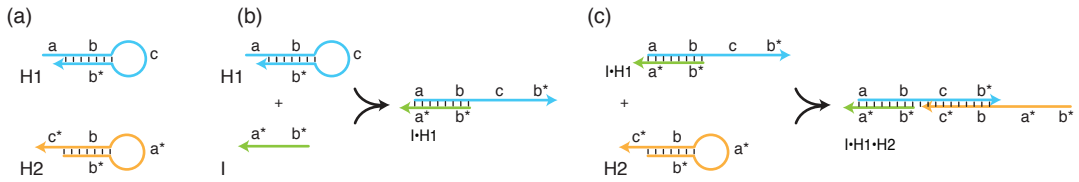


Figure 5.1: **HCR polymer formations schematic.** Letters marked with * are complementary to the corresponding unmarked letter (e.g a is complementary to a*). (a) Hairpins H1 and H2 are kinetically trapped in monomer state. (b) In the presence of initiator I hairpin H1 binds to the initiator and undergoes a conformation change, exposing the loop stem, forming complex I·H1. (c) The exposed loop and stem of hairpin H1 enable hairpin H2 to bind, forming a complex I·H1·H2 and exposing its loop and stem. The open form of H2 is identical to the initiator, enabling to another H1 hairpin to bind. A hybridization chain reaction occurs which forms a nicked double-stranded alternating H1 and H2 hairpins.

5.2 HCR as a detection method for northern blots

An HCR polymer can be used to detect and amplify the signal of a northern blot probe. Figure 5.2 depicts an overview of the northern blot procedure with HCR amplification as the signal detection step. To use HCR for detection, the probe design needs to be altered from that of "standard" probes. Traditional probes have a region complementary to the target RNA as well as a detection moiety such as a radioactive or hapten label. For HCR detection, the detection moiety of the probe is changed into a nucleotide sequence which is an HCR initiator. Once the probe is hybridized to its target, an HCR initiator "tail" is left unbound to the membrane (Figure 5.2, probe hybridization). After unbound probe is washed, HCR amplifier hairpins are added to the blot and an HCR polymer is grown off of each target-bound probe. Each amplifier hairpin is labeled, therefore each polymer contains multiple labels resulting in signal amplification (Figure 5.2, HCR amplification). The unbound hairpins are then washed and the signal is detected accordingly.

The HCR amplifiers are orthogonal. By using a different initiator per target-specific probe, a different polymer can be grown off multiple types of targets (Figure 5.2, red, blue and yellow polymers). The signal can be further amplified by growing multiple HCR

polymers per target. This can be achieved by using multiple probes per target, each with the same initiator sequence. For detection, any molecule used for standard northern blot probes can be used. Because HCR systems are orthogonal, multiplexing is inherent. Thus, as long as the probes are selective enough, multiple targets can be detected simultaneously. For multiplexing, distinct fluorescent labels should be used for each HCR amplifier.

5.3 Results

5.3.1 HCR "dot blot"

We used a dot blot to show that HCR can be used to detect a target mRNA in a blot format. To that end, we used a dot-blot approach, where an mRNA of interest is spotted in known amounts onto a membrane and detected using fluorescent HCR. Choi et al. [26] have previously used probes designed to target enhanced green fluorescent protein (EGFP) for *in situ* hybridization using HCR amplification. One of these probes was used for the dot blot since it was pre-validated and a plasmid for *in vitro* transcription of EGFP was present in the lab. This probe is 81 nucleotides long with a 50-nucleotide gene specific region, a 5-base spacer and a 26-nucleotide HCR initiator. Mild blotting conditions were used to promote probe binding since no hybridization to other targets was expected. Blotting conditions were adapted from *mir*Vana miRNA isolation kit (Ambion).

Figure 5.3 demonstrates that HCR polymers can indeed be grown off an mRNA-bound probe attached to a positively charged nylon membrane. Figure 5.3(a) suggests a sensitivity estimated at three femtomoles. A second blot was performed using smaller amounts of spotted mRNA to see whether the sensitivity achieved in Figure 5.3(a) is due to full consumption of amplifiers or due to a sensitivity limit. A slight improvement in signal was observed when less RNA was spotted (Figure 5.3(b)). Taken together, both blots suggest a detection limit on the order of one to three femtomoles. This is less sensitive than commercial buffers such as ULTRAhyb Ultrasensitive Hybridization Buffer (Ambion), which

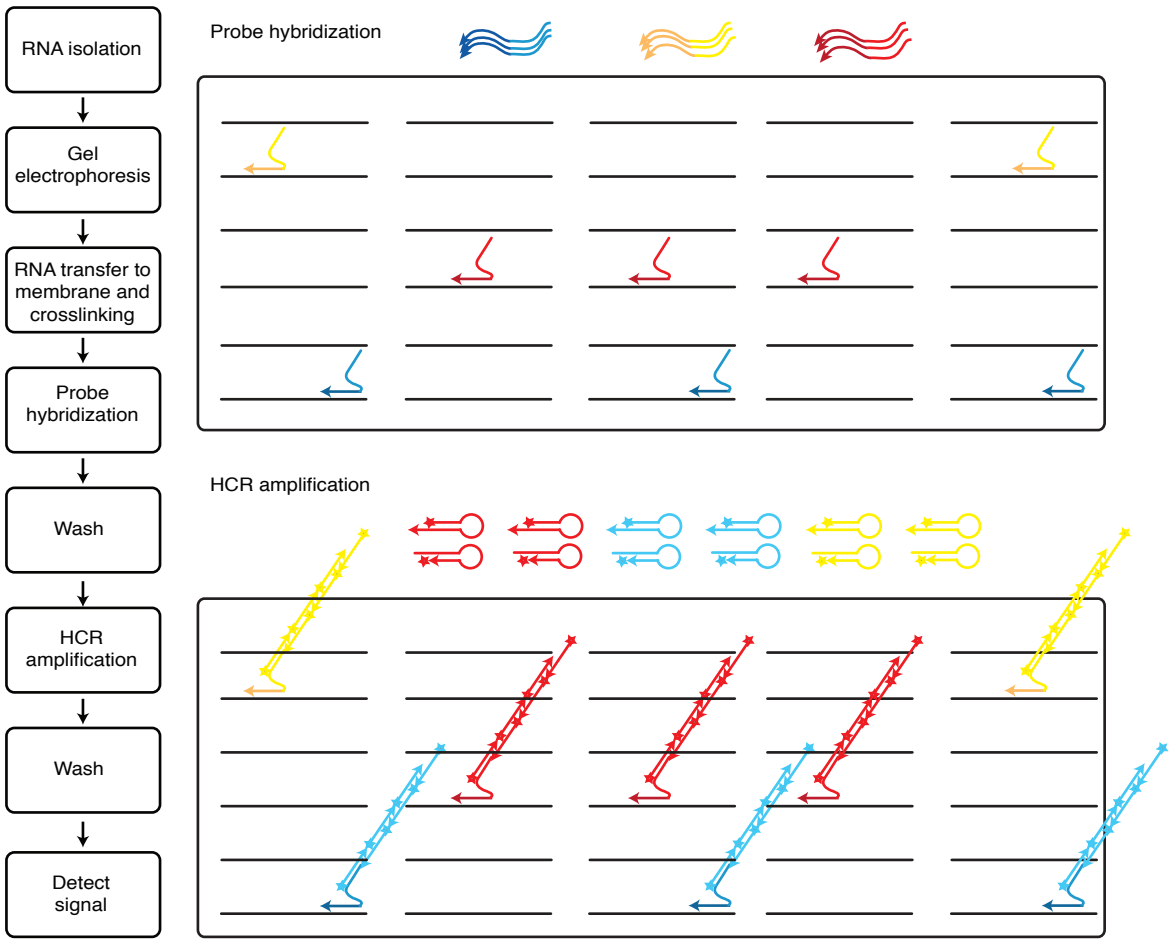


Figure 5.2: **Northern blot detection via HCR schematic.** Each probe contains an HCR initiator sequence. A different HCR polymer can be grown off each target, according to the initiator sequence. The two colors of each probe symbol the target binding sequence and the initiator sequence. Blue hairpins can form a polymer off blue initiator sequences etc. Arrows on probes and hairpins represent the 3′ end. Stars represent the detection moiety.

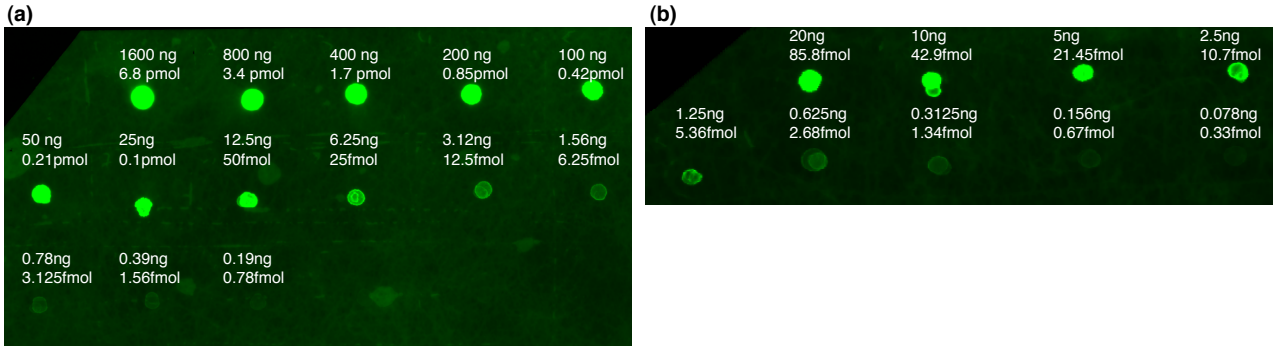


Figure 5.3: **EGFP "dot blot" detection via HCR.** Serial dilutions of *in vitro* transcribed EGFP mRNA were spotted onto a Nytran SPC (Whatman) membrane. RNA was cross-linked to the membrane by baking for 2 hours at 80°C. Blotting was done in mild conditions according to the *mir*Vana miRNA blotting procedure (see Materials and methods). The EGFP probes (see Table 5.2.)were used at a 2nM concentration. Probe detection was carried out using a 10nM (each) amplifier solution in hybridization buffer. Amplifiers were snap cooled prior to hybridization and amplification step was carried out over night at room temperature in the dark (system A1, see Table 5.3). The amount of moles per spot were estimated based on a EGFP mRNA molecular weight of 223069.33 gr/mole. (a) Two-fold dilution series starting with 1600 nanogram EGFP mRNA. (b) Two-fold dilution series starting with 20 nanogram EGFP mRNA.

advertise the detection of 10,000 molecules (attomolar range). This sensitivity is based on the use of very pure and highly radioactive probes with prolonged exposure times (days) and 100% efficiency in probe hybridization to the target — conditions which are not standardly achieved. It is possible that the use of a different buffer such as ULTRAhyb might improve the hybridization conditions.

5.3.2 mRNA blots

5.3.2.1 Specificity and blotting conditions

To check for specificity of the assay (probe binding and non-specific HCR amplification) and to examine blotting conditions, destabilized enhanced green fluorescent protein (d2EGFP) was chosen as the detection target. This gene was chosen mainly due to its visible phenotype

in tissue culture and due to the availability of cell lines with and without d2EGFP. In addition, in our lab we have multiple probes against EGFP which have been validated *in situ*. These probes also match up with the d2EGFP sequence. Probes are 81 nucleotides in total with a 50-nucleotide gene specific region, a 5-nucleotide spacer and a 26-nucleotide HCR initiator sequence.

mRNA blots from total RNA were attempted using the same conditions for the dot blot. These conditions were not stringent enough and led to non-specific probe biding, most likely to ribosomal RNA (293A vs. 293 d2EGFP lanes in Figure 5.4(a)). Blotting was also attempted with the same hybridization and amplification buffer conditions that were used for *in situ* HCR amplification [26]. These conditions were not compatible for blotting and resulted in no signal (including for *in vitro* transcribed mRNA; data not shown). Finally, probe hybridization and HCR amplification were attempted using ULTRAhyb hybridization buffer (Ambion). Prior to RNA transfer to the membrane, the gel was stained with SYBRGold to verify the integrity of the RNA (Figure 5.4(b)). Staining of ribosomal RNA demonstrates that the RNA has not been degraded; the amount of *in vitro* transcribed mRNA (30ng) is not visible by SYBRGold staining in these conditions (control mRNA lane, Figure 5.4(b)). Figure 5.4(c) demonstrates that an HCR signal is obtained only for lanes containing either *in vitro* transcribed EGFP mRNA (control mRNA lane) or total RNA from d2EGFP expressing cells but not for total RNA from non-expressing 293A cells. The lack of signal in total RNA from 293A cells is due to the lack of d2EGFP mRNA and not due to mRNA degradation as evident by Figure 5.4(b). Thus, these conditions are suitable for specific mRNA detection and HCR amplification.

5.3.2.2 Multiplexed detection of endogenous targets

So far we have shown that HCR can detect over-expressed d2EGFP mRNA. We now demonstrate HCR's ability to detect endogenous levels of mRNA expression using glyceraldehyde-3-phosphate dehydrogenase (GAPDH). We chose GAPDH due to its relatively high expres-

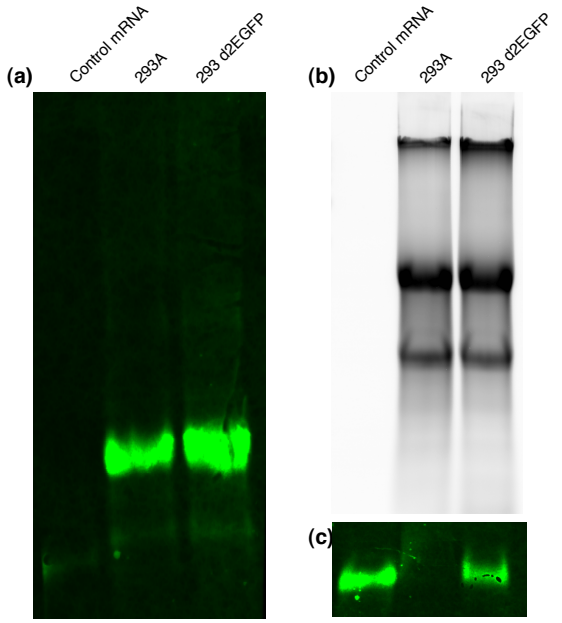


Figure 5.4: **Specific d2EGFP detection via HCR.** *In vitro* transcribed EGFP mRNA and total RNA extracted from 293A or 293 d2EGFP cells were separated on 1% denaturing agarose and transferred onto a Nytran SPC (Whatman) membrane. Following cross-linking and pre-hybridization a probe against d2EGFP (see Table 5.2) was added and hybridized overnight. The gel was washed three times before HCR amplification. The signal was amplified overnight using 14.3nM of each amplifier hairpin (system A3, see Table 5.3) labeled with Alexa 532. The membrane was washed 3 times prior to imaging. (a) Alexa 532 signal from blotted membrane. Blotting was done in mild conditions according to the *mir*Vana miRNA blotting procedure (see Materials and methods sectoin). 5.7nM of EGFP probe were used for hybridization. (b) SYBRGold staining of electrophorated RNA prior to transfer demonstrating the RNA is intact (30ng control mRNA and 22.5μg total RNA). (c) Alexa 532 signal from blotted membrane. Blotting conditions: pre-hybridization and hybridization with 9.17nM probe in ULTRAhyb. Two 5 minute washes at room temperature in low stringency buffer followed by a 5 minute wash in high stringency buffer at 55°C (NorthernMax kit, Ambion). After amplification the high stringency wash was done at 45°C.

sion level and because it is commonly used as a control in northern blot experiments. To demonstrate that the detected GAPDH mRNA runs corresponding to its size, a separate HCR system was used to detect a single-stranded RNA ladder (NEB). Probes are 81 nucleotides long with a 50-nucleotide gene-specific region, a 5-nucleotide spacer and a 26-nucleotide HCR initiator sequence. An HCR system labeled with Alexa 488 was used to detect an ssRNA ladder probe and an HCR system labeled with Alexa 647 was used to detect GAPDH probes. The expected length of GAPDH mRNA is 1401 nucleotides (NCBI accession NM_002046); a band corresponding to a size between 1000–2000 nucleotides is present in the Alexa 647 channel in Figure 5.5(a), suggesting that this is GAPDH. Both total RNA as well as total mRNA were used for this assay; total mRNA was used to control for non-specific binding to ribosomal RNA since it should be mostly depleted of ribosomal RNA. Figure 5.5(b) demonstrates that the 18S unit of the ribosomal RNA runs similar in size (1869 nucleotides, NCBI accession NR_003286) to the 2000nt marker, further supporting that the observed detected band is GAPDH and not ribosomal RNA.

To further validate that the observed signal is due to GAPDH detection and not ribosomal RNA (rRNA), a probe specific for 18S rRNA [27] was used in combination with probes against GAPDH and a single-stranded RNA (ssRNA) ladder. The 18S rRNA probe has a 20-nucleotide binding region whereas the other probes have a 50-nucleotide binding region. All probes have a binding region to the sequence of interest as well as an initiator for HCR. GAPDH, 18S rRNA and an ssRNA ladder were all detected using three orthogonal HCR systems; each system was labeled with a different fluorophore. Figure 5.6 demonstrates that a signal obtained for GAPDH (red) indeed runs lower than the 18S rRNA (green signal). In addition, it is observed that while the MicroPoly(A) purist kit does a good job of removing most of the 18S rRNA, some still remains as is observed by the green band in the total mRNA lane.

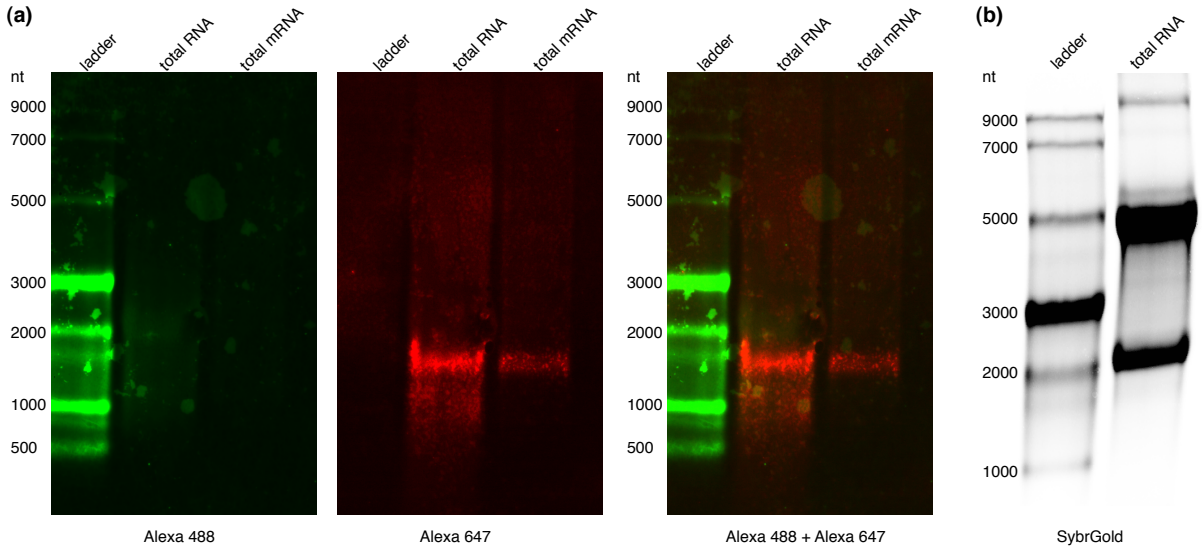


Figure 5.5: **Multiplexed GAPDH and ssRNA ladder detection via HCR.** Blotting was performed according to the mRNA blotting procedure (see Materials and methods). (a) Total RNA (34.5μg, Trizol extraction) or total mRNA (940ng, MicroPoly(A) purist (Ambion)) extracted from 293A cells were separated on 1% denaturing agarose and transferred onto a BrightStar-PLUS SPC (Whatman) membrane. As a size marker, 1.25μg ssRNA ladder (NEB) was used. Following cross-linking and pre-hybridization, five probes against GAPDH (each with an initiator for HCR system A1) were added to a 1.8nM final concentration (each), to detect the ladder, 16.3nM probe with an initiator for HCR system A2 was used. HCR amplification was performed at 20nM of each amplifier hairpin. Alexa 488 amplifiers bind to probes against ssRNA ladder, Alexa 647 amplifiers bind to probes against GAPDH. (b) SYBRGold staining of total RNA and ssRNA ladder demonstrates the RNA is intact and the size of ribosomal RNA bands. For a list of probes and HCR amplifier systems see Tables 5.2 and 5.3.

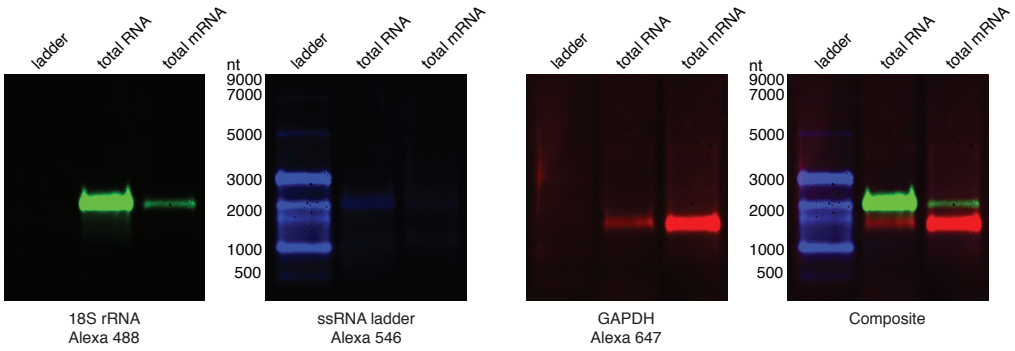


Figure 5.6: **Multiplexed detection of GAPDH, 18S rRNA and ssRNA ladder via HCR.** Total RNA (8μg, *mir*Vana extraction) or total mRNA (920ng, MicroPoly(A) purist (Ambion)) extracted from 293A cells were separated on 1% denaturing agarose and transferred onto a BrightStar Plus (Ambion) membrane. As a size marker 1μg, ssRNA ladder (NEB) was used. Blotting was performed according to the mRNA blotting procedure (see Materials and methods section). Five probes against GAPDH (each with an initiator for HCR system A1) were added to a 8.5nM final concentration (each). To detect the ladder, 8.5nM probe with an initiator for HCR system A2 was used. To detect 18S rRNA, 10nM probe with an initiator for HCR system A6 was used. HCR amplification was performed at 28.5nM of each amplifier hairpin. Alexa 488 amplifiers bind to the 18S rRNA probe, Alexa 546 amplifiers bind to the ssRNA ladder probe, Alexa 647 amplifiers bind to the GAPDH probes. For a list of probes and HCR amplifier systems see Tables 5.2 and 5.3.

5.3.3 miRNA blots

Our next goal was to use HCR to detect small RNAs in the range of 19–60 nucleotides (such as siRNAs, miRNAs, piwi-interacting RNAs (piRNAs) [28–30] and our own scRNAs). The detection of small RNAs is more challenging than that of longer mRNAs. Their abundance is typically low and combined with the loss of small RNAs during the extraction process, the blotting procedure requires increased sensitivity. Additionally, due to their short sequence, only one probe can be used per target which may reduce the amount of labels per target. Due to these challenges, a sensitive multiplexed assay using HCR is advantageous.

5.3.3.1 Blotting conditions for miRNAs

To examine miRNA detection using HCR, we chose hsa-miR-16a as the miRNA target, U6 small nuclear 1 (RNU6-1) as an internal control, and a microRNA marker (NEB) as a size marker. The U6 gene is 106 nucleotides long and is often used as a small-RNA loading control. Probes for the miRNA and the ladder are the full complement to the target. For the ladder, the complement to the 17 nucleotides that are present in all three bands was used as the probe. Due to initial difficulties in detecting microRNAs (data not shown), two additional controls were used: a synthetic miR16a target was used to check that the probe and blotting conditions can bind the miR-16a target, and in addition, the synthetic target was spiked into the sample prior to RNA extraction to check for the ability to recover miRNAs.

Two blotting conditions were used, one at room temperature as suggested by the *mir*Vana protocol (for a detailed protocol see Materials and methods section; Figure 5.7(a)) and one at 55°C probe hybridization and 45°C HCR amplification as observed suitable for *in situ* [26] and mRNA blotting (Figure 5.7(b)). Figure 5.7 demonstrates that blotting at room temperature leads to non-specific HCR amplification as is evident both for the U6 probe and the miR-16a probe, while blotting in elevated temperatures results in specific detection (Figure 5.7(a) vs. Figure 5.7(b), blue and red channels). The miR-16a probe is

able to amplify its target. However, a signal was not observed for total RNA or samples enriched for small RNAs. The low signal obtained from the spiked sample suggests that the extraction procedure may result in partial loss of miRNAs or that the cross-linking process is not sufficient. Nevertheless, the possibility that miR-16a is not expressed, or expressed at non-detectable levels cannot be ruled out.

Real-time PCR was used to verify the expression level of miR-16a in HEK293A cells. The expression of miR-16a is estimated to be 4.5 ± 0.08 [1]-fold lower than that of U6. Once it was verified that miR-16a is present in the HEK293 cell line, we used a FAM-labeled LNA probe (Exiqon) against miR-16a to examine the northern blot procedure. LNA probes are commonly used as probes in northern blot detection and are considered to have increased sensitivity and specificity compared to DNA and RNA probes [9, 31]. The labeled LNA probe and a synthetic target were used as controls in the blot. Blotting conditions according to the *mir*Vana protocol did not yield any signal, including for the control groups (data not shown). Given that using ULTRAhyb for the mRNA blots significantly improved the blotting conditions, we tried blotting with ULTRAhyb-Oligo (Ambion), which is designed for short probes. Figure 5.8(a) demonstrates that while the blotting conditions are sufficient for detection of the synthetic target and probe controls, they are not suitable for detection of endogenous levels of miR-16a.

Next, we examined the cross-linking conditions. Both short-wave UV light and baking cross-link RNA to the membrane through interactions with the RNA bases. Cross-linking of bases within the RNA target, especially for short targets, reduces their availability to interact with the probe. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) cross-linking is thought to work via the $5'$ terminal phosphate of the small RNA and results in an immobilized RNA which has more free bases to interact with a probe. The use of EDC has improved the detection of small RNAs by a minimum of 20-fold [32]. By altering the cross-linking method to EDC, miR-16a was detected in total RNA, total RNA spiked with

[1] For calculation of the error see Materials and methods section.

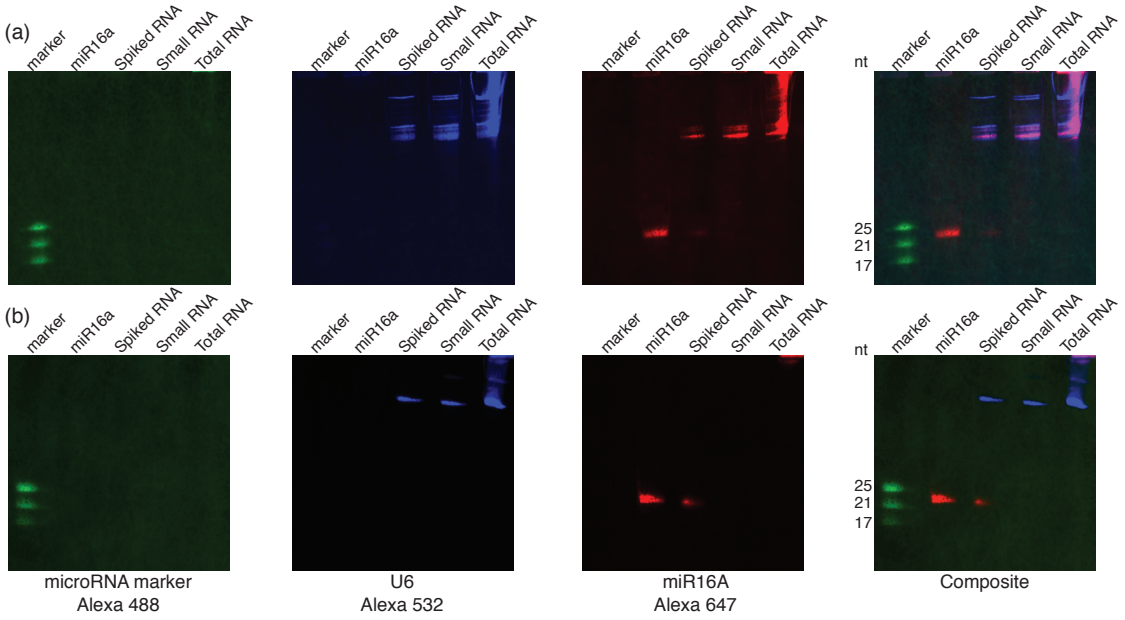


Figure 5.7: **Multiplexed detection of synthetic miR-16a target, endogenous U6 RNA and microRNA ladder via HCR.** Extraction of small RNAs was done from HEK293 d2EGFP cells according to the *mir*Vana enrichment procedure for small RNAs (Ambion). For the sample spiked with a synthetic target, the target was added prior to extraction. 60ng microRNA marker, 0.5pmol synthetic miR-16a target, 720ng of small RNA extraction with a spiked synthetic marker, 975ng of small RNA extraction and 22.5μg total RNA extracted with ZR RNA MiniPrep (Zymo Research) were separated on a 15% denaturing polyacrylamide gel. Blotting was carried out according to the *mir*Vana miRNA blotting procedure (see Materials and methods section). Probes were used at a 5nM concentration each. Amplification was done at 10nM. (a) Hybridization, wash and HCR amplification at room temperature. (b) Hybridization and wash at 55°C, HCR amplification and wash at 45°C. Probes: microRNA ladder A2 initiator, miR-16a A1 initiator, U6 A5 initiator. Amplifiers: A1 Alexa 647, A2 Alexa 488, A5 Alexa 546. For a list of probes and HCR amplifier systems see Tables 5.2 and 5.3.

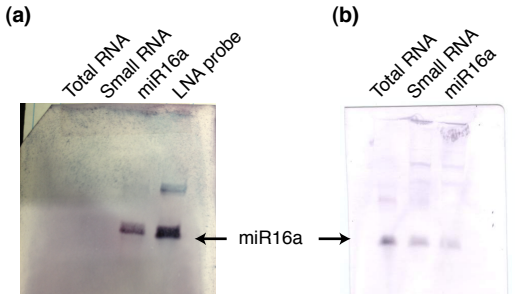


Figure 5.8: **Detection of endogenous miR-16a target using a FAM-labeled LNA probe.** Extraction of total RNA or small RNAs was done from HEK293A or HEK293 d2EGFP cells according to the relevant *mir*Vana protocol (Ambion). Detection of the probe was done using 1.5mg/ml alkaline phosphatase anti-fluorescein antibody (Vector Laboratories) followed by BCIP/NBT chromogenic detection. (a) 15μg total RNA, 1.6μg small RNAs, 0.5pmol synthetic miR-16a and 5pmol FAM-labeled LNA probe were blotted onto a positively charged nylon membrane. Cross-linking was done by baking for 30 minutes at 80°C. Probe concentration for hybridization was 1.25nM. (b) 34μg total RNA, 18.5μg total RNA spiked with 0.1pmol synthetic miR-16a (after extraction), 0.1pmol synthetic miR-16a were blotted onto a positively charged nylon membrane. Cross-linking was done using EDC. Probe concentration for hybridization was 3.3nM.

a synthetic miR-16a target and a synthetic miR-16a target using a FAM-labeled LNA probe (Figure 5.8(b)). Taken together, these results suggest that the use of ULTRAhyb-Oligo in combination with EDC cross-linking are good conditions for detection of endogenous miRNAs.

5.3.3.2 Multiple HCR-initiator probes

The low abundance of miRNAs makes them a difficult target to detect. While the signal of mRNAs can be amplified by the use of multiple probes per target (thus growing multiple HCR polymers per target), the short length of small RNA targets allows use of only one probe per target. In order to try and increase the signal generated per probe, multiple initiators can be attached to an HCR probe, allowing one probe to facilitate the polymerization of multiple polymers. Figure 5.9 depicts this strategy using double-initiator (DI)

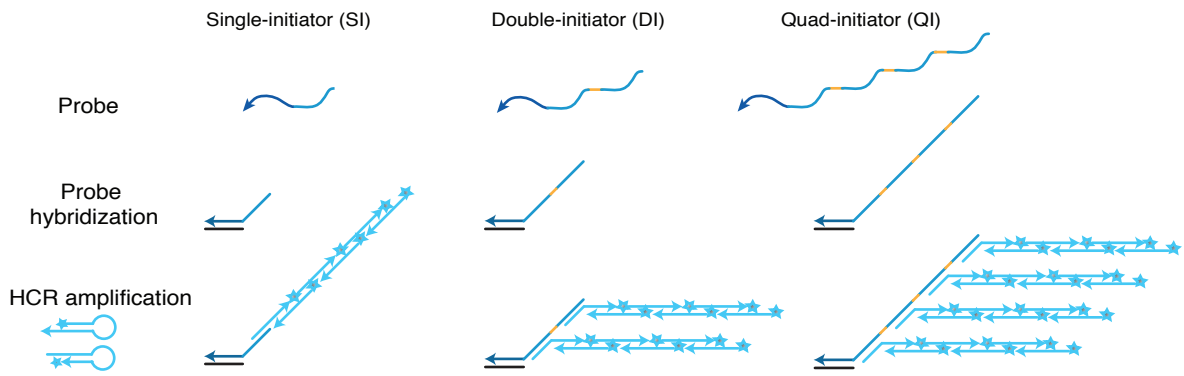
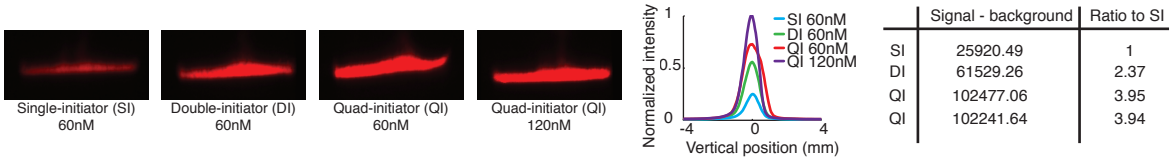


Figure 5.9: **HCR-based detection using probes with multiple initiators schematic.** Target (black), probe region complementary to the target (dark blue), initiator sequence (turquoise), spacer sequence between initiators (orange), amplifier hairpins (light blue) and detection moiety (star).

and quad-initiator (QI) probes. Figure 5.10 demonstrates that increasing the number of initiators increases the observed signal as expected. It is yet to be determined whether this relationship is linear. Compared to the signal of a single initiator (SI probe), a DI probe increased the signal by 1.84±0.56 and a QI probe increased the signal by 3.07±0.98. Signal increase represents the average increase in signal across three independent experiments; errors represent the standard error of the mean. Amplification was done at 60nM for each amplifier hairpin for SI, DI and QI probes. To verify that this regime is in excess, an additional sample with 120nM amplifier hairpins was included with a QI probe. The signal did not significantly change by doubling the amount of amplifier suggesting that 60nM is a sufficient concentration.

5.3.3.3 Multiplexed miRNA detection using HCR

By using EDC cross-linking in combination with ULTRAhyb-Oligo for probe hybridization, a DI probe and HCR amplification, it is possible to detect endogenous levels of miR-16a in 293A total RNA (Figure 5.11). We next examined whether it is possible to detect two



	Signal - background	Ratio to SI
SI	25920.49	1
DI	61529.26	2.37
QI	102477.06	3.95
QI	102241.64	3.94

Figure 5.10: **HCR-based detection using probes with single, double and quad initiators.** One pmol synthetic miR-16a target were blotted using 20nM of single-, double-, or quad-initiator probes for HCR system A1. Amplification was carried out at 60nM or 120nM (indicated in the figure) with Alexa 546-labeled amplifiers. The histogram depicts the normalized intensity of each band. For a detailed method see miRNA blotting procedure with ULTRAhyb-Oligo in the Materials and methods section. For a list of probes and HCR amplifier systems see Tables 5.2 and 5.3.

different microRNAs simultaneously. The challenge in this case is that both miRNAs would be present at the same location on the membrane. To this end, we have chosen miR-21 as the second miRNA target. Based on data obtained in small RNA library sequencing, we expect this microRNA to be expressed at very low levels in HEK cells and expressed at similar levels to miR-16a in the glioblastoma cell line U87MG [33]. The use of RNA extracted from both cell lines can be used as a positive and negative control on a blot.

Figure 5.12 demonstrates that a microRNA ladder, U6 RNA, miR-16a and miR-21 can all be detected simultaneously in a northern blot procedure; these targets could be detected from as little as 1μg total RNA (Figure 5.12(c) and (d)). In order to detect all four targets, the microRNA ladder, as well as one probe, had to use the same label due to technical reasons related to lab equipment. The amplifier systems were different and only the labeling (Alexa dye) is identical. Detection of two microRNAs is not limited to small (endogenous) amounts of target; 0.5pmol (each) of miR-16a and miR-21 can be detected using multiplexed blotting (Figure 5.13).

Based on small-RNA library sequencing data [33], we expected miR-16a and miR-21 to be expressed at similar levels in U87MG cells. However, detection of miR-16a was surprisingly low (Figure 5.12). This is not likely due to the blotting procedure, as is evident from Figure 5.13. It might be due to a variation in the glioblstoma cell line used

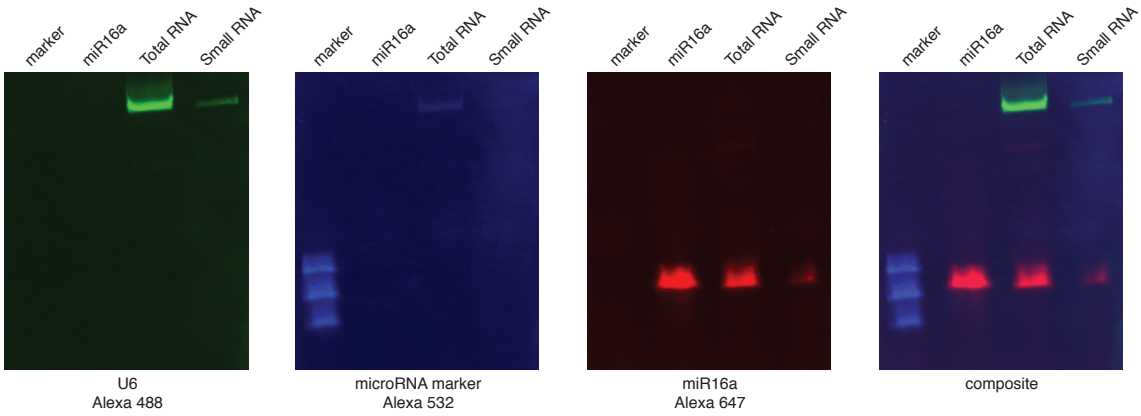


Figure 5.11: **Multiplexed HCR-based detection of endogenous miR-16a.** 12ng microRNA marker, 0.1pmol synthetic miR-16a, 25.8μg total RNA and 364ng extracted RNA enriched for small RNAs were used for blotting. Hybridize with miR-16a-DI probe, U6 short SI probe, microRNA marker probe DI at 5.7nM. After hybridization and HCR amplification the membrane was washed twice in 2×SSC 0.1% SDS at room temperature for 5 minutes followed by a 5-minute wash in 0.1×SSC 0.1% SDS at 37°C. For a list of probes and HCR amplifier systems see Tables 5.2 and 5.3.

or due to bias in small RNA library sequencing [34, 35]. The expression of each microRNA relative to U6 was examined using qRT-PCR in both cell lines. Preliminary data show that expression of miR-16a in U87MG cells is indeed lower than expression of miR-21 and in the HEK293A cell line, miR-21 is expressed at lower levels than miR-16a (data not shown).

5.3.3.4 Detection limit of HCR-based miRNA northern blot detection

Kim et al. [10] have recently reported that by using digoxigenin-labeled LNA probes in combination with EDC cross-linking, detection of 0.05 femtomol miRNA was achieved; this is comparable to radioactive labeling. We next examined whether HCR detection can compare in sensitivity. Using serial dilutions of synthetic miR-16a, HCR detection with a QI probe using Alexa 647-labeled amplifiers has a sensitivity limit of 0.1 femtomol (Figure 5.14(a)). Radioactive labeling with ^{32}P is most commonly used due to its high sensitivity. Whereas using a DI probe with ^{32}P labeled HCR amplifiers maintains a sensitivity of 0.1

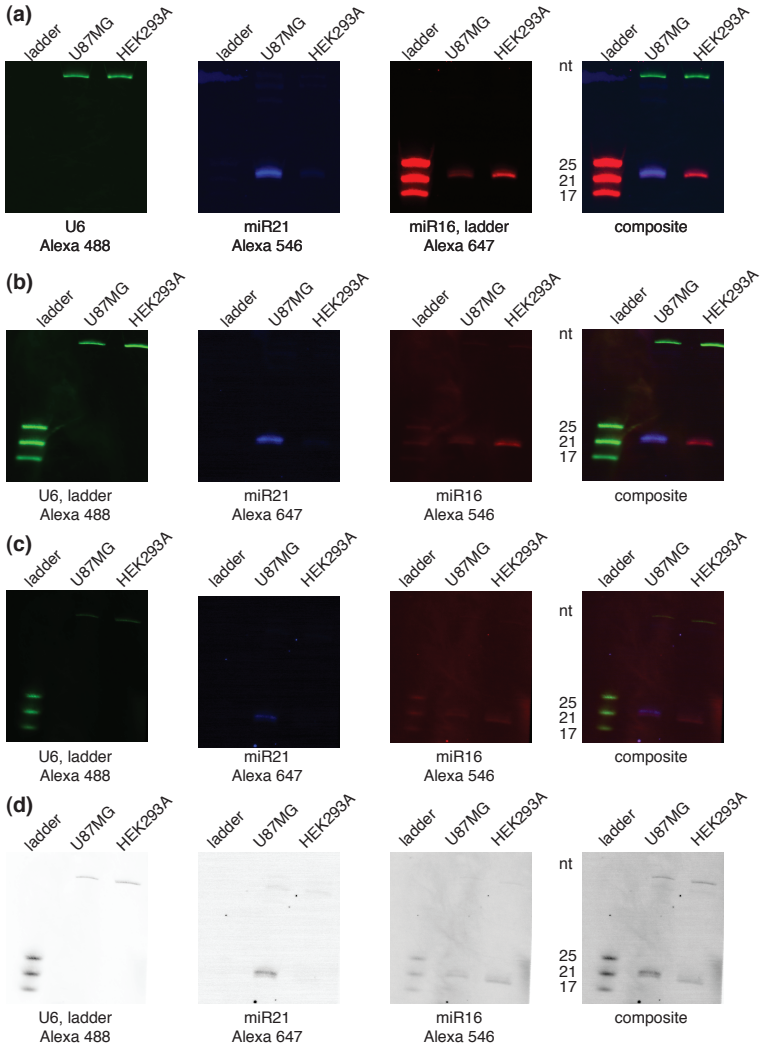


Figure 5.12: **Multiplexed HCR-based detection of endogenous miR-16a, miR-21 and U6.** All blots were performed using 20nM probes and 60nM of each amplifier hairpin. For detailed methods see miRNA blotting procedure with ULTRAhyb-Oligo in the Materials and methods section. Probes: ladder DI A2, $U6_{short}$ SI A5, miR-16a QI A1, miR-21 QI A3. (a) 5μg total RNA, 24ng microRNA marker. Amplifiers: A1 Alexa 647, A2 Alexa 647, A3 Alexa 546, A5 Alexa 488. (b) 3μg total RNA, 18ng microRNA marker. Amplifiers: A1 Alexa 546, A2 Alexa 488, A3 Alexa 647, A5 Alexa 488. (c) 1μg total RNA, 18ng microRNA marker. Slightly less than 20nM probe for the microRNA ladder were used. Amplifiers: same as panel C. (d) Greyscale representation of panel (c). For a list of probes and HCR amplifier systems see Tables 5.2 and 5.3.

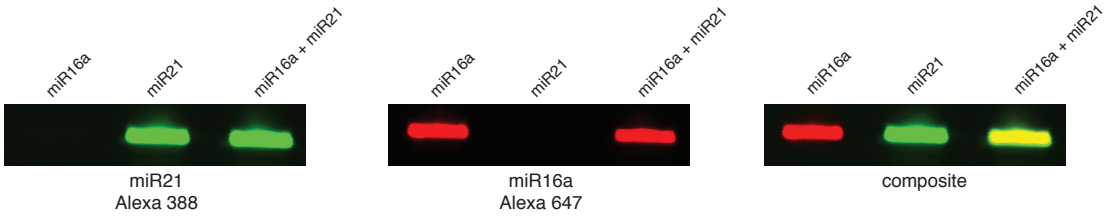


Figure 5.13: **Multiplexed HCR-based detection of miR-16a and miR-21.** 0.5pmol of synthetic microRNA targets were blotted using 20nM probes and 50nM of each amplifier hairpin, for a detailed method see miRNA blotting procedure with ULTRAhyb-Oligo in the Materials and methods section. Probes: miR-16a DI A1, miR-21 DI A3. Amplifiers: A1 Alexa 647, A3 Alexa 488. For a list of probes and HCR amplifier systems see Tables 5.2 and 5.3.

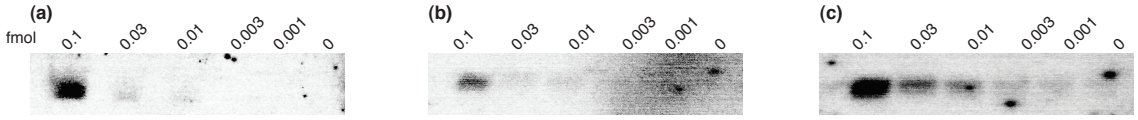
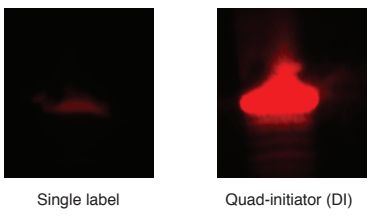


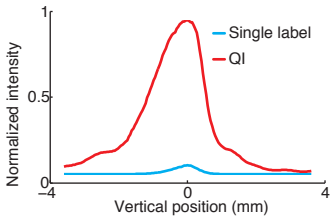
Figure 5.14: **Sensitivity of HCR-based detection.** Serial dilutions of 5′ phosphorylated synthetic miR-16a targets blotted using ULTRAhyb-Oligo (see Materials and methods section). (a) 20nM QI A1 probe, 60nM A1 amplifiers labeled with Alexa 647. (b) 20nM DI A1 probe, 0.5×10^6cpm/ml of each ^{32}P amplifier hairpin (10^6cpm/ml total). (c) 13.5nM QI A1 probe, 0.5×10^6cpm/ml of each ^{32}P amplifier hairpin. For a list of probes and HCR amplifier systems see Tables 5.2 and 5.3.

femtomol, by using radioactive HCR with a QI, probe the detection limit improves to 0.01 femtomol, a five-fold sensitivity increase compared to that reported in the literature (Figure 5.14(b) and (c)). The QI sensitivity limit is maintained when probe concentration is increased from 13.5nM to 20nM (data not shown).

5.3.3.5 Estimation of polymer length

Finally, we wanted to know how many hairpins (fluorphores) are present in each HCR polymer. To estimate the number of hairpins present, the signal obtained from one probe labeled with an Alexa dye was compared to the signal obtained from an HCR polymer





	Signal - background	Ratio to Single label
Single label	307245.61	1
QI	10342656.18	33.66

Figure 5.15: **Estimation of HCR polymer length.** 2pmol 5′ phosphorylated synthetic miR-16a target were blotted using20nM miR-16a antisense probe labeled with Alexa 647 or with a QI A1 HCR probe. HCR detection was carried out using 60nM A1 Alexa 647 labeled amplifiers. The blot of the single labeled probe was blot underwent the exact same procedure as the HCR blot without the addition of amplifiers. The histogram depicts the normalized intensity of each band produced. For a list of probes and HCR amplifier systems see Tables 5.2 and 5.3.

initiated by a QI probe. The signal obtained for the QI probe was roughly 33 times higher than that of a probe with one label. The signal obtained for a QI probe should be composed of four HCR polymers, estimating a polymer length of about eight hairpins per polymer (Figure 5.15). This estimate is in agreement with estimation of the polymer length of an SI probe against d2EGFP (data not shown).

5.4 Discussion

We have presented the use of a hybridization chain reaction as a method for northern blot detection. We have shown that HCR is suitable to detect both long targets such as mRNAs as well as short targets such as microRNAs. In HCR detection, the target probe contains an additional "tail" which serves as an HCR initiator. During the detection step, a polymer is grown off of each bound probe. Each amplifier hairpin is labeled with a detection moiety (fluorescent dye or radio-label) for signal readout. The HCR polymers are orthogonal using a different HCR initiator on each probe each target has a unique polymer attached to it.

The main advantage of HCR over current detection methods (e.g ^{32}P, biotin etc.) is that it allows for parallel multiplexing. By labeling each HCR hairpin (and therefore

polymer) with a specific fluorophore, each polymer is visualized distinctly and specifically with its own target. Thus, multiple targets can be probed and detected simultaneously on the same blot. Not only does this significantly save time, but it also reduces the risk and complications involved with stripping and re-probing blots. Many possibilities exist for the generation of orthogonal HCR polymers. The limitation in the number of targets that can be detected simultaneously is technical and depends on the spectral channels of the imaging device.

A limitation of fluorescent HCR is that fluorescence is typically less sensitive than radioactive labeling [21] with ^{32}P. In this work, we show a detection limit of about 0.1 femtomol miR-16a using a quad-initiator RNA HCR probe, whereas the reported literature suggest a detection limit of 0.05 femtomol using a ^{32}P or digoxigenin-labeled LNA probe [10]. However, if HCR labeling is switched to ^{32}P, then the detection limit of HCR becomes 0.01 femtomol, a 5-fold improvement relative to the literature.

Further improvements to sensitivity can also be made by changing the nucleotide composition of the probe to 2′-OMe or LNA. The main drawback is that these may not allow for multiple initiators; due to synthesis technicalities they cannot be transcribed, and are more expensive.

Alternatively, improvements to sensitivity can be made by increasing polymer length or increasing the number of polymers per target. Our work suggests that each polymer is composed of approximately eight hairpins, while gel studies for the same polymers suggest a mean length of 20 hairpins [26]. Further optimization of hybridization conditions may be needed to grow longer polymers. For long RNA targets, multiple probes can be used to grow multiple polymers per target and thus improve sensitivity. This strategy is not possible with short miRNA targets. To overcome this, we have designed probes with multiple initiators. This way, one probe can have multiple HCR-polymers attached to it therefore leading to an increase in signal. Indeed, we have shown that multiple-initiator probes lead to a stronger signal when compared to a single-initiator probe. A further

increase in signal may be obtained by optimizing the spacer length between the initiator sequences.

Other strategies based on nucleic acids structures have been used to obtain signal amplification. Mainly, the use of DNA dendrimers and branched DNA (bDNA) assays have been used as the amplification method [36–42]. These assays were mostly focused on signal amplification for ISH, microarrays, miRNA detection in microtiter plates and plasma. Although these strategies can potentially be used for signal amplification in blotting methods, to the best of our knowledge, this is not common practice.

The work presented here is not limited to northern blot detection of multiple targets. It may also be used to detect the same target mRNA redundantly in two channels using different HCR systems. This can assist in validating the specificity of probes. More broadly, HCR can be used as a detection method for many other hybridization-based techniques such as Southern blot, array formats and possibly for point-of-care detection. This work provides a stepping stone toward achieving sensitive, multiplexed detection in diverse *in vitro* settings using HCR.

5.5 Materials and methods

***in vitro* transcription**. pCS2plus-EGFP plasmid was a generous gift from Dr. Le Trinh. The plasmid was linearized with NotI prior to SP6 transcription. Transcription was carried out using AmpliScribe SP6 high yield transcription kit (Epicenter), according to the manufacturer. The transcribed RNA was purified using Qiagen's RNeasy mini kit according to the manufacturer, on-column DNaseI digestion was performed. RNA was quantified using A_{260} absorbance on a NanoDrop8000 (Thermo Scientific).

Snap cool. Amplifier hairpins were snap cooled by heating them to 95°C for 90 seconds followed by a $\geq$30 minute incubation at room temperature in the dark.

Cell lines. HEK293A cells were purchased from Invitrogen (catalong #R705-07), HEK293 d2EGFP cells were a generous gift from Dr. Chase Beisel. The destabilized EGFP sequence comes from pd2EGFP-1 plasmid (Clonetech, PT3205-5 catalog #6008-1). Cells were maintained at 37°C 5% CO_2 in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen).

RNA extraction. Total RNA was extracted either with Trizol reagent (Ambion), ZR RNA MiniPrep (Zymo Research) or *mir*Vana miRNA isolation kit (Ambion).

mRNA blotting procedure. Denaturing formaldehyde/MOPS agarose gel was prepared with 1×NorthernMax denaturing gel buffer (Ambion) according to the manufacturer. Prior to loading, total RNA was mixed 1:1 (vol/vol) with formamide and heated to 65°C for 15 minutes. The gel was run in 1×MOPS buffer (Ambion) at 55Volts for 2–3 hours. The gel was washed four times in water prior to transfer. A positively charged nylon membrane (BrightStar-Plus (Ambion) or Nytran-SPC (Whatman)) was pre-wet in water and then equilibrated 5 minutes in 20×SSC. RNA transfer to the membrane by "downward transfer" in 20×SSC for 3–4 hours. The RNA was cross-linked to the membrane by baking at 80°C for two hours between two 3MM Whatman filter sheets. The membrane was pre-hybridized at 65°C for at least 30 minutes in ULTRAhyb (Ambion). Probes were added to the pre-hybridization solution and hybridization was carried out overnight at 55°C. The blots were

washed twice in 2×SSC 0.1% SDS for 5 minutes at room temperature followed by a 5-minute 0.1×SSC 0.1%SDS wash at 55°C unless otherwise specified. The membrane was then pre-hybridized again in ULTRAhyb followed by addition of snap cooled HCR amplifier hairpins. Amplification was carried out overnight at 45°C in the dark. Prior to imaging, the blots were washed twice in 2×SSC 0.1% SDS for 5 minutes at room temperature followed by a 5-minute 0.1×SSC 0.1%SDS wash at 45°C unless otherwise specified.

***mir*Vana miRNA blotting procedure.** Pre-hybridization was carried out for at least an hour at 65°C in 6×SSC, 10×Denhardt's solution, 0.2%SDS. Probes were hybridized to the membrane overnight at room temperature in 6×SSC, 5×Denhardt's solution, 0.2% SDS unless otherwise specified. The membrane was washed at room temperature three times in 6XSSC, 0.2% SDS prior to HCR amplification unless otherwise specified. Amplification was carried out at room temperature overnight in the dark unless otherwise specified. After amplification the membrane was washed as indicated above.

miRNA blotting procedure with ULTRAhyb-Oligo. Samples were run on a 15% denaturing polyacrylamide gel at 300 volts for 25–30 minutes. When total RNA was used, the gel was pre-run prior to loading the RNA. For electrophoresis, the samples were mixed 1:1(vol/vol) with formamide and heated to 95°C for 5 minutes or to 65°C for 15 minutes . A positively charged nylon membrane (Roche) or NytranSPC (Whatman) was pre-wet in water and then equilibrated 5 minutes in 0.5×TBE prior to transfer. Semi-dry transfer in 0.5×TBE was used at 0.8mA to 2mA per square centimeter of gel for 45 minutes to 2 hours using a Panther semidry electroblotter (Owl separation systems). Unless otherwise specified, the RNA was cross-linked to the membrane using EDC (see below). Pre-hybridization, hybridization and HCR amplification were carried out in ULTRAhyb-Oligo (Ambion) at 37°C unless otherwise specified. The membrane was washed 2–3 times in 2×SSC 0.5% SDS or 2×SSC 0.1% SDS for 5–15 minutes after probe hybridization and after amplification.

BCIP/NBT chromogenic detection. Detection was done using the protocol and solu-

tions in the Biotin Chromogenic Detection kit (Thermo Scientific). Alternatively, blocking was done in 1X casein solution (Vector Laboratories) and washed in 1×PBS 1% Tween20.

EDC cross-linking. 245μl of 12.5M 1-methylimidazole (Alfa Aesar) were added to 9ml of RNAse-free water. pH was adjusted to 8 using 1M HCl. 0.753gr of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; Sigma or Bio-Rad) were added to the solution and the volume was adjusted to 24ml with water. The EDC solution was used to saturate a 3MM Whatman chromatography paper. The membrane was placed on the saturated sheet with the RNA side facing up and both the membrane and the Whatman paper were wrapped in Saran. Cross-linking was done by a 1–2 hour incubation at 60°C. Following cross-linking, the membrane was washed with water to remove excess cross-linking solution.

Imaging. Membranes were imaged on an FLA-5100 imaging system (Fuji Photo Film). Radioactive membranes were exposed onto an image plate (Fujifilm type BAS-MS) and scanned using the IP-S mode at 600V. Fluorescent membranes were scanned using the following settings:

Dye	Excitation	Filter
Alexa 488	473 nm	BP 530 ± 10nm
Alexa 532	532 nm	BP 570 ± 10nm
Alexa 647	635 nm	LP 665 nm

Table 5.1: Excitation lasers and emission filters used.

Quantification. MultiGauge software (Fujifilm Life Sciences) was used to quantify gels using the quantitative analysis with a profile feature.

5′ end labeling. HCR amplifier hairpins were 5′ end labeled with [$\gamma-^{32}$P] ATP (10mCi/ml, MP Biomedicals) using T4 polynucleotide kinase (New England Biolabs). Unincorporated [$\gamma-^{32}$P] ATP were removed by spin column chromatography using Illustra MicroSpin G-25 columns (GE Healthcare) according to the manufacturer. Counts were measured on a Beckman LS-5000TD Liquid Scintillation Counter.

qPCR analysis. RNA for qPCR was extracted using TaqMan MicroRNA Cells-to-Ct kit (Ambion) according to the manufacturer. cDNA was synthesized using TaqMan microRNA

reverse transcription kit (Ambion). Real-time PCR reaction was prepared using TaqMan Universal PCR Master Mix, No AmpErase UNG and carried out in a CFX96 (Bio-Rad). TaqMan primer pairs used were purchased from Applied Biosystems: hsa-miR-16 (catalog #000391), hsa-miR-21 (catalog #000397) and U6 snRNA (catalog #001973).

Expression of microRNA relative to U6 was done by simplifying the Pfaffl method [43] to include only one sample while correcting for qPCR efficiency of each reaction using the equation,

$$\text{Ratio} = \frac{U6}{microRNA} = \frac{{E_{microRNA}}^{C_{t(microRNA)}}}{{E_{U6}}^{C_{t(U6)}}}. \tag{5.1}$$

Three biological replicates were included and three technical replicates per biological replicate (9 data points total).

The efficiency of the reaction was calculated using the following equation $\text{E}=10^{\frac{-1}{slope}}$ where the *slope* was calculated based on a serial 10-fold dilution of a cDNA sample. Reactions with Ct values higher than 36 were not used for the analysis. Each dilution series was analyzed in triplicate.

To account for the uncertainty in the Ct values on the Ratio (equation (5.1)), we use the simple propagation of errors. The uncertainty, σ_f of a quantity $f(x, y)$, that is a function of variables x and y with known uncertainties σ_x and σ_y, is given by,

$$\sigma_f = \sqrt{\left(\frac{\partial f}{\partial x}\sigma_x\right)^2 + \left(\frac{\partial f}{\partial y}\sigma_y\right)^2}. \tag{5.2}$$

Thus, the uncertainty in the Ratio (equation (5.1)), given the uncertainties $\sigma_{C_{t(microRNA)}}$ and $\sigma_{C_{t(U6)}}$ is given by,

$$\sigma_{\text{R}atio} = \text{R}atio\sqrt{\left(log(E_{microRNA})\sigma_{C_{t(microRNA)}}\right)^2 + \left(log(E_{U6})\sigma_{C_{t(U6)}}\right)^2}. \tag{5.3}$$

Probe	HCR system	Sequence

EGFP SI	A1	CCGAAUACAAAGCAUCAACGACUAGA *AAAAA***GUUCUUCUGCUUGUCGGC** **CAUGAUAUAGACGUUGUGGCUG** **UUGUAGUUGU**
EGFP SI	A3	GACUACUGAUAACUGGAUUGCCUUAG *AAUUU***GUUCUUCUGCUUGUCGGC** **CAUGAUAUAGACGUUGUGGCUG** **UUGUAGUUGU**
GAPDH 1 SI	A1	CCGAAUACAAAGCAUCAACGACUAGA *AAAAA***AAAAGAAGAUGCGGCUGA** **CUGUCGAACAGGAGGAGCAGAG** **AGCGAAGCGG**
GAPDH 2 SI	A1	CCGAAUACAAAGCAUCAACGACUAGA *AAAAA***UCCGUUGACUCCGACCUU** **CACCUUCCCCAUGGUGUCUGAG** **CGAUGUGGCU**
GAPDH 3 SI	A1	CCGAAUACAAAGCAUCAACGACUAGA *AAAAA***CCCGUUCUCAGCCUUGAC** **GGUGCCAUGGAAUUUGCCAUGG** **GUGGAAUCAU**
GAPDH 4 SI	A1	CCGAAUACAAAGCAUCAACGACUAGA *AAAAA***UUCCACGAUACCAAAGUU** **GUCAUGGAUGACCUUGGCCAGG** **GGUGCUAAGC**
GAPDH 5 SI	A1	CCGAAUACAAAGCAUCAACGACUAGA *AAAAA***UCGCUGUUGAAGUCAGAG** **GAGACCACCUGGUGCUCAGUGU**

		AGCCCAGGAU
18S rRNA SI	A6	CCACAUACCAUCAGACCAGACUAGAC
		*AAAUA***CGGAACUACGACGGUAUC**
		UG
Low range ssRNA ladder SI	A2	GACCCUAAGCAUACAUCGUCCUUCAU
		*UUUUU***CUCGACGAAGACUCCC**
ssRNA ladder SI	A2	GACCCUAAGCAUACAUCGUCCUUCA
		*UUUUUU***AUUUUUUCCAAGACAU**
		CUUCCAGUCGCUGGCGCUUGG
		GGUACCAUCAGCU
miRNA ladder SI	A2	GACCCUAAGCAUACAUCGUCCUUCAU
		*UUUUU***AUCUCAACCAGCCACUG**
miRNA ladder DI	A2	GACCCUAAGCAUACAUCGUCCUUCAU
		*UUUUU*GACCCUAAGCAUACAUCGUCC
		UUCAU*UUUUU***AUCUCAACCAGCC**
		ACUG
hsa-miR-16a SI	A1	CCGAAUACAAAGCAUCAACGACUAGA
		*AAAAA***CGCCAAUAUUUACGUGCU**
		GCUA
hsa-miR-16a DI	A1	CCGAAUACAAAGCAUCAACGACUAGA
		*AAAAA*CCGAAUACAAAGCAUCAACGA
		CUAGA*AAAAA***CGCCAAUAUUUACG**
		UGCUGCUA
hsa-miR-16a QI	A1	CCGAAUACAAAGCAUCAACGACUAGA
		*AAAAA*CCGAAUACAAAGCAUCAACGA
		CUAGA*AAAAA*CCGAAUACAAAGCAUC
		AACGACUAGA*AAAAA*CCGAAUACAAA

		GCAUCAACGACUAGA*AAAAA***CGCCA**
		AUAUUUACGUGCUGCUA
hsa-miR-21 QI	A3	GACUACUGAUAACUGGAUUGCCUUAG
		*AAUUU*GACUACUGAUAACUGGAUUGC
		CUUAG*AAUUU*GACUACUGAUAACUGG
		AUUGCCUUAG*AAUUU*GACUACUGAUA
		ACUGGAUUGCCUUAG*AAUUU***UCAAC**
		AUCAGUCUGAUAAGCUA
U6 SI	A5	UACGCCCUAAGAAUCCGAACCCUAUG
		*AAAUA***CGUUCCAAUUUUAGUAUA**
		UGUGCUGCCGAAGCGA
U6 short SI	A5	UACGCCCUAAGAAUCCGAACCCUAUG
		*AAAUA***UGUGCUGCCGAAGCGA**

Table 5.2: List of probes used. In bold is the target binding sequence, italicized is the 5nt spacer, the rest is the initiator(s) sequence. Sequences are listed 5′ to 3′

HCR system	Hairpin	Sequence
A1	H1	UCUAGUCGUUGAUGCUUUGUAUUCGGCGACAGAUAAC CGAAUACAAAGCAUC /C9-dye-3′/
A1	H2	/5′-dye-C12/ CCGAAUACAAAGCAUCAACGACUAGAGAU GCUUUGUAUUCGGUUAUCUGUCG
A2	H1	AUGAAGGACGAUGUAUGCUUAGGGUCGACUUCCAUAG ACCCUAAGCAUACAU /C9-dye-3′/
A2	H2	/5′-dye-C12/ GACCCUAAGCAUACAUCGUCCUUCAUAUG UAUGCUUAGGGUCUAUGGAAGUC
A3	H1	CUAAGGCAAUCCAGUUAUCAGUAGUCUGACACGACUG ACUACUGAUAACUGG /C9-dye-3′/
A3	H2	/5′-dye-C12/ GACUACUGAUAACUGGAUUGCCUUAGCCA GUUAUCAGUAGUCAGUCGUGUCA
A5	H1	CAUAGGGUUCGGAUUCUUAGGGCGUAGCAGCAUCAAU ACGCCCUAAGAAUCC /C9-dye-3′/
A5	H2	/5′-dye-C12/ UACGCCCUAAGAAUCCGAACCCUAUGGGA UUCUUAGGGCGUAUUGAUGCUGC
A6	H1	GUCUAGUCUGGUCUGAUGGUAUGUGGACAAUCCUAGC CACAUACCAUCAGAC /C9-dye-3′/
A6	H2	/5′-dye-C12/ CCACAUACCAUCAGACCAGACUAGACGUCU GAUGGUAUGUGGCUAGGAUUGU

Table 5.3: List of HCR amplifiers used. Sequences are listed 5′ to 3′ .

Bibliography

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