miRNA Detection by Ligation and Amplification of Complementary DNA oligos Using SplintR® ligase

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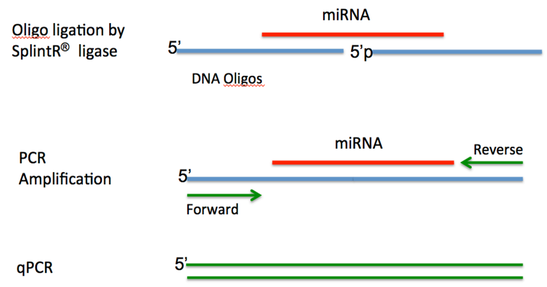
Abstract

Ligation of two adjacent DNA oligonucleotides splinted by RNA has historically been difficult to achieve. We have discovered that SplintR® ligase (Chlorella virus DNA ligase) is much more efficient in this ligation than T4 DNA ligase, which was traditionally used for this application. When these ligases were compared using the same RNA:DNA substrates the SplintR® enzyme achieved complete ligation with 10X less ligase and was 15X faster than T4 DNA ligase [1].

We have taken advantage of this efficient RNA splinted ligation and developed an extremely sensitive and specific miRNA detection protocol. In this method, two DNA oligos, splinted by a miRNA, were ligated and amplified by qPCR in presence of a dual labeled DNA probe. Using this method, we can detect miR122 from less than 1 ng of rat liver total RNA. Further more we found that efficient ligation can be achieved with only a 4-6 base pair overlap between one of the DNA oligos and the miRNA splint. The SplintR® ligase can discriminate a single nucleotide mismatch between DNA oligos and miRNA splint. We designed DNA oligos that were specific for individual members of the mammalian let-7 family and were able to detect specific let-7 isoforms. The efficient and specific ligation of RNA:DNA hybrids by the SplintR® ligase should allow it to be used in a wide range of RNA detection methods for both cellular and viral RNAs.

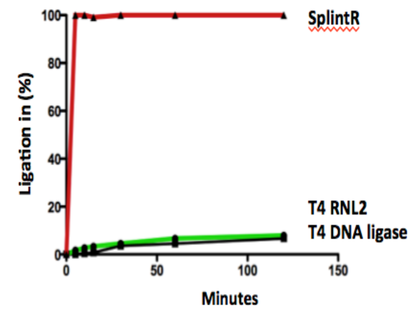
[1] Lohman et al. Nucleic Acids Res. advanced access, Nov. 6, 2013. PMID:24203707.

miRNA Detection by Splint Ligation



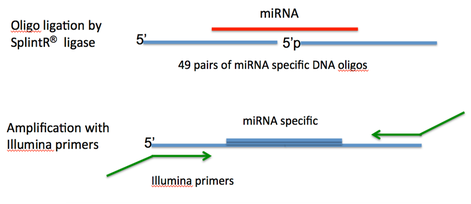
Ligation based method for miRNA detection.
Two DNA oligos, complementary to a specific miRNA, are hybridized and ligated by SplintR ligase. The probes can be amplified and detected by qPCR.

SplintR ligase vs T4 DNA ligase and T4 RNA ligase 2



Time course RNA splinted DNA ligation.
Two DNA probes complementary to miR-122 were ligated with three different ligases: SplintR ligase, T4 DNA ligase and T4 RNA ligase 2. Only one probe was FAM labeled. The extend of ligation was determined by capillary electrophoresis (CE) fragments analysis.

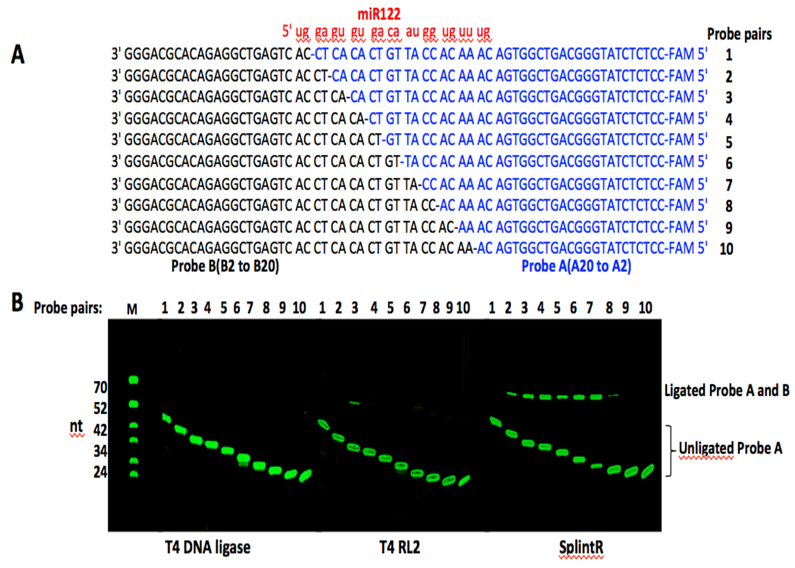
Multiplex miRNA detection by Sequencing



	A	B	C	D
1	2747519	CAAACACCATTGTCACACTCCACTAG	miR-122	
2	1920107	CATCACCTTCCTTCTCCTCCACTAGC	miR-765	
3	805920	ACCCCTATCACGATTAGCATTAACTA	miR-155-5p	
4	768197	CCTCCAGTCCTTGCACCGAGACCTAG	miR-678	
5	768065	AGAGCTACAGTGCTTCATCTCACTAG	miR-143	
6	484269	CCTGGCACACAGTAGGACCTTCACTA	miR-493-3p	
7	472639	ACGCTCAAATGTCGCAGCACTTTCTA	miR-372-3p	
8	462981	CTACCTGCACTGTAAGCACTTTGCTA	miR-17-5p	
9	315662	CGTACGCTATACGGTCTACTACTAGC	miR-411-5p	
10	311028	CCTACGTTCCATAGTCTACCACTAGC	miR-379-5p	
11	193170	CACAAGTTCGGATCTACGGGTTCTAG	mir-100-5p	
12	186006	TCTACGGGTTCTAGCAGCCTGACATC	no match	
13	161376	TTCCCATGCCCTATACCTCTCTAGCA	miR-202-3p	
14	115034	CTGTTCCTGCTGAACTGAGCCACTAG	miR-24-3p	
15	96902	ACCAGGTTCCACCCCAGCAGGCCTAG	miR-370	
16	83354	TTGTCTAACCAGTCACCTGTTCTAGC	mir-522	
17	75582	CAGCTATGCCAGCATCTTGCCTCTAG	miR-31-5p	
18	57253	TCAACATCAGTCTGATAAGCTACTAG	miR-21-5p	
19	43130	GCTCTAAGAAAGCCACACTCTAGCAG	miR-644a-3p	
20	42389	ACACTCTAAAGGGAACCATTTTCTAG	miR-522-3p	
21	30447	AAGAACAATGCCTTACTGAGTACTAG	miR-201-5p	
22	29157	CCTGTCACACAGTAGGACCTTCACTA	miR-493-3p	1 mismatch
23	28263	TCACAGGTTAAAGGGTCTCAGGGACT	miR-125a-5p	
24	26908	CTACCTGCACTGTAAGCACTTTTCTA	miR-106a-5p	
25	26170	CGCCAATATTTACGTGCTGCTACTAG	miR-16-5p	
26	22766	ACAGAGAGCTTGCCCTTGTATACTAG	mir-381-3p	
27	22335	TCCATCATCAAAACAAATGGAGTCTA	miR-136-5p	
28	17010	AAGAACAATGCCTTTGTGTGATCTAG	miR-377-3p	half site
29	16608	CCTGGCACACAGTATGACCTTCACTA	miR-493-3p	
30	16478	ACAATCCTAGCCTTCACTAGCAGCCT	no match	
31	16320	AACACTGATTTCAAATGGTGCTACTA	miR-29b-3p	
32	13253	AAAGAGACCGGTTCACTGTGACTAGC	miR-128-3p	
33	12260	CCTCCAGTCTAGCCACACTCTAGCAG	no match	
34	11292	CACAAGTTCGGATCTACGTGTTCTAG	miR-100-5p	1 mismatch
35	10912	ACCGACCGACCGATCGACCGACTAGC	miR-341-3p	
36	10644	TCATCATTACCAGGCAGTATTACTAG	miR-200b-3p	
37	10340	GAGGGAGGGGCTGATAAGCTACTAGCA	no match	
38	8227	ACGCTCAAGATGTGCTGCTACTAGCA	miR-15b-5p	5 nt short
39	7873	ACAAAAGTTGCCTTTGTGTGATCTAG	miR-377-3p	
40	6921	TTCCCATTCCCTATACCTCTCTAGCA	miR-202-3p	1 mismatch

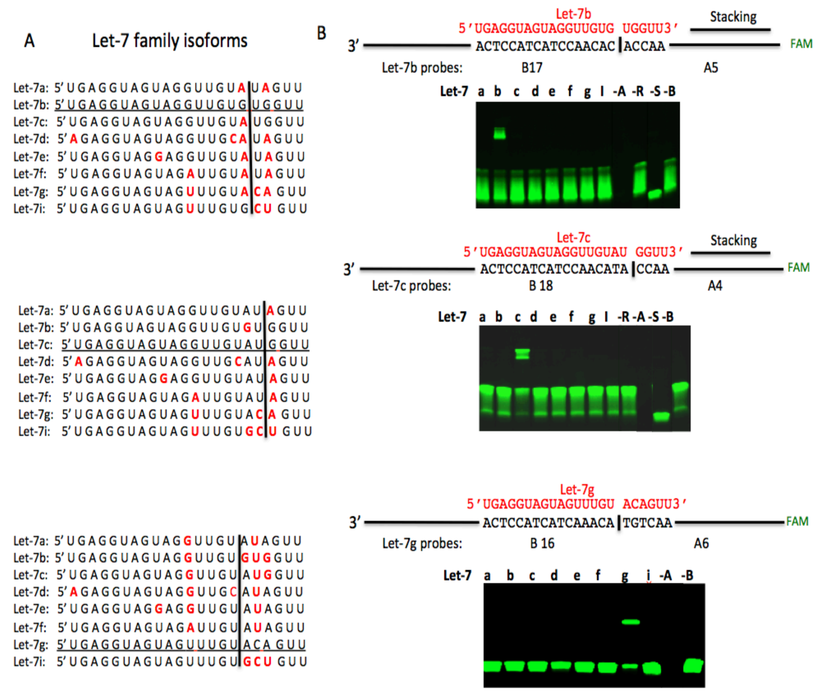
Multiplex detection of miRNAs using 49 pairs of DNA oligos.
49 pairs of miRNA-specific DNA oligos were hybridized to a library of 960 synthetic miRNAs (Miltenyi Biotec), ligated by SplintR ligase, amplified and sequenced on a MiSeq machine. A ten fold weight excess of yeast RNA was added to the hybridization to mimic a biological sample. The total MiSeq reads are shown in column A, sequences in column B and the miRNA identity in column C. Over 80% of the reads are the correct ligation products.

Small overlap required for RNA splint ligation



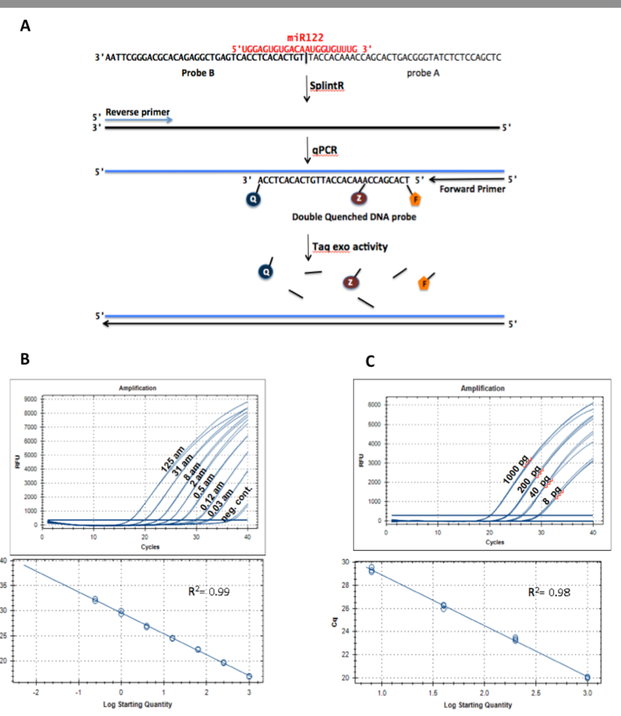
Identifying minimum RNA:DNA hybrid required for RNA splint ligation.
(A) Pairs of DNA oligonucleotides complementary to miR-122 were used to determine the minimum DNA:RNA overlap required for ligation. The miR-122 sequence (red) is complementary to probe A (blue) and probe B (black). Probe A and probe B are designed to scan the miRNA sequence in two base increments. Probe A has a 5' FAM label and probe B has a 5' phosphate that allows ligation. (B) A denaturing gel of the ligated products shows that only a 4 to 6 base overlap is required for SplintR ligation of the two DNA oligos. The top band on the gel is the ligated product. The FAM labeled DNA oligo is in excess to the miRNA so ligated and unligated products are observed.

Specific detection of Let-7 isoforms



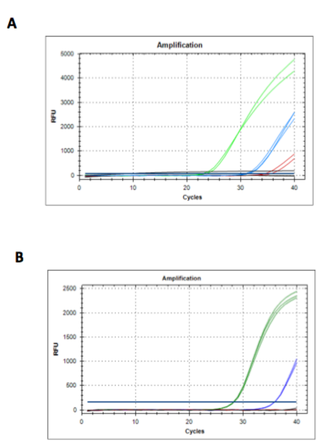
RNA splint ligation detects single nucleotide difference in let-7 family.
Specific DNA probes were designed for let-7b, let-7c and let-7g. The probes were individually hybridized to eight different let-7 miRNAs and ligated with SplintR ligase. For short overlaps a stacking oligo was used to enhance stability. Only the FAM labeled probe A is seen on the gels. Controls are: no probe A (-A), no probe B (-B), no ligase (-R), no stacking oligo
(-S). To the left of each gel are the sequences of each let-7 isoform. Nucleotide differences between the probes and miRNA are shown in red. The ligation junction is indicated by a vertical black line.

High sensitivity - miR122 detection in rat liver RNA



Quantitative detection of miR-122 in Rat liver total RNA by splint ligation.
DNA oligos complementary to miR-122 were ligated by SplintR ligase and then amplified and detected by qPCR. Amplification was performed with NEB OneTaq hot start 2X master mix. (A) Amplification was detected with a miR-122 specific double quenched FAM labeled probe from IDT . (B) A miR-122 standard curve using an RNA oligo and at least 1,000X excess of non-specific yeast RNA. (C) Different amounts of rat liver total RNA were used for miR-122 detection The SplintR-qPCR assay has sub-attomole sensitivity for miR-122.

Comparison of SplintR to TaqMan for miRNA detection



Rat Liver Total RNA	Cq value	Detection miR-122 SplintR/Taqman
1 ng	23	32X
0.2 ng	31	32X
0.04 ng	35	NA
No miRNA	NA	NA
1 ng	28	
0.2 ng	36	
0.04 ng	NA	
No miRNA	NA	

Comparison of SplintR ligation and TaqMan for qPCR detection of miR-122.
Three different concentrations of rat liver RNA; 1 ng (green), 0.2 ng (blue), 0.04 ng/µL (red) and no RNA (black) were used for qPCR detection of miR-122. The qPCR probe is described in the panel to the left. (A) This panel shows the qPCR traces for the SplintR method. (B) TaqMan detection of miR-122 used DNA hairpin primed cDNA synthesis followed by qPCR detection (Life Technologies). A comparison of the sensitivity of the two methods is shown at the right side of the figure. NA stands for not applicable because there was no amplification above the threshold value.

Summary

- Chlorella virus DNA ligase, SplintR® ligase, is much more efficient than either T4 DNA ligase or T4 RNA ligase 2 in ligation of DNA oligos hybridized to a miRNA splint.
- SplintR ligase requires only a 4 to 6 base DNA:RNA overlap for ligation.
- The SplintR method coupled with qPCR can detect miRNAs in biological samples in the sub-attomole amounts.
- The SplintR ligase method is about 30 fold more sensitive than the TaqMan method, that uses cDNA synthesis of the miRNA.
- Multiplex detection of miRNAs can be achieved by the simultaneous ligation of 49 pairs of miRNA-specific DNA probes followed by NextGen DNA sequencing.
- Single base differences in members of the let-7 family can be detected by SplintR ligation.

Reference
Lohman GJ, Zhang Y, Zhelkovsky AM, Cantor EJ, Evans TC Jr, Nucleic Acids Res. 2014 Feb 1;42(3):1831-44. **Efficient DNA ligation in DNA-RNA hybrid helices by Chlorella virus DNA ligase**