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A sensitive multiplex assay for piRNA expression

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Abstract

PIWI-interacting RNAs (piRNAs) are new class of small RNAs specifically expressed in male germ cells. It is known to bind to PIWI class of Argonaute proteins-Mili, Miwi, and Miwi-2. To decipher the mechanism of piRNA function, here we report a real-time PCR based multiplex assay for piRNA expression. Firstly, we showed that the assay specifically detects piRNA expression in adult testis, consistent with Northern blot result. Then we confirmed that we can simultaneously detect at least eight piRNAs expression using only 10pg total RNA –equivalent to RNA amount of a single cell. This is five to six orders more sensitive than corresponding Northern blot. Finally we use this assay to analyze eight piRNAs expression in primordial germ cells (PGCs) in genital ridges from E12.5, the time point piRNA-binding protein Mili starts to express in PGCs. This multiplex piRNA assay can be further expanded to assay a few hundred of piRNAs simultaneously from as little as total RNA of a single cell, that will help to understand the mechanism and function of piRNAs for germ cell development.

INTRODUCTION

PIWI-interacting RNAs (piRNAs) are a new class of small non-coding RNAs important for germ cell development (1-3). The size of piRNAs is between 26nt and 31nt, which is distinct from microRNAs of 21-23nt lengths. The processing of piRNAs seems also distinct from microRNAs because there are no hairpin structures similar to microRNAs being found for piRNAs. In drosophila, it is expressed in both male and female germ cells, whereas in mammals, it is specifically expressed in male germ cells (3). They bind to MIWI family proteins- Mili (Piwi2), Miwi (Piwi1), and Miwi2 (Piwi4), and RecQ1 to form piRNA complex (piRC) to function (4-6). RecQ1 is an adenosine triphosphate (ATP)-dependent DNA helicase and is homologous to Qde-3 of Neurospora, which is important for gene silencing. Most mammalian piRNAs are clustered together at limited numbers of regions in the genome (4-8). Mammalian piRNAs from a same cluster usually distribute on only one genomic strand in non-overlapping manner. PiRNAs are usually expressed from syntenic regions in human, mouse, and rat, but their sequence is distinct and not conservative (4-6). Mili and Miwi2 knockout mice showed spermatogenesis arrest at the zygotene stage and cannot progress to early pachytene stage (9, 10). The spermatogenesis of Miwi knockout mice arrested at the round spermatid stage (11, 12). All these three knockout mice are male infertile indicating that piRNAs have an essential role for spermatogenesis (9-12). One of the potential roles of piRNAs for spermatogenesis is to repress transposons because both Mili and Miwi2 knockout mice showed strong upregulation of transposons LINE-1 and IAP

in male germ cells (10, 13). Until now, tens of thousands of Miwi- or Mili-binding piRNAs have been cloned from mammalian testis (4-8).

The gold method to detect piRNA expression is Northern Blot (4-7). Yet it is laborious and needs 10 - 60ug of total RNAs to detect each piRNA's expression. An easier and more sensitive detection method for piRNAs is heavily needed. Here we developed a multiplex detection method based on real-time PCR to profiling multiple piRNAs from 10pg RNAs that is about amount of RNAs in a single cell. To increase the specificity, we used barcode reverse primers to specifically convert corresponding piRNAs into cDNAs, pre-amplify them with universal reverse primer, and finally detect individual ones by barcode TaqMan probe directed real-time PCR. It combines the power of stem-loop-structured primer technique and barcode primer technique and can specifically detect multiple piRNAs expression using tiny amount of total RNAs (14).

MATERIALS AND METHODS

Isolation of total RNAs

Genital ridges of E12.5, E14.5, E16.5, and E18.5 embryos were dissected under dissection microscope. Male and female genital ridges were discriminated by morphology. Testis and other tissues were dissected from neonatal and adult mice. MirVana miRNA isolation kit (Ambion) was used to isolate total RNA (Including piRNAs) according to the manufacturer's protocol. For the experiment to determine the specificity of the multiplex piRNA assay, we use 10pg total RNA from each tissue. For the experiment to check piRNA expression in PGCs and testis, we use 1ng total RNAs from genital ridges or testis at different stages starting from E12.5.

Reverse transcription

The RT reaction volume is 5ul. The RT reactions were performed following the manufacture's suggestions using ABI high capacity cDNA archive kit (CN: 4322171). 0.5ul of 10× cDNA archiving kit buffer, 0.5ul 8-plex barcode piRNA reverse transcription primers (50nM of each one), 0.25ul 100mM dNTP, 0.065ul 20U/ul RNase inhibitor, and 0.335ul MMLV reverse transcriptase were added into RT reaction. We used pulsed RT reaction condition to increase RT efficiency and reduce non-specific interactions between primers for different piRNAs. There is no amplification of the piRNA cDNAs at this step. The RT reaction condition was: First, 16°C for 30min, then 60 cycles at 20°C for 30sec, 42°C for 30sec, and 50°C for 1sec, and finally, 85°C for 5min to inactivate Reverse Transcriptase.

Pre-amplification reaction

All of the RT product (5ul) was used as template for a 25ul pre-amplification reaction. 0.25ul 8-plex barcode piRNA forward primers (5uM of each one), 12.5ul 2X TaqMan Universal Master Mix (ABI), 1ul 100mM dNTP, 2ul 25mM MgCl₂, 1.25ul 100uM Universal Reverse Primer, 1.25ul 5U/ul AmpliGold Taq (ABI) were used for each Pre-amplification reaction. The reaction condition is: 95°C for 10min, 55°C for 2min, followed by 18 cycles of 95°C for 1sec and 65°C for 1min.

Real-time PCR reaction

2ul of 1:40 diluted Pre-amplification product was used for a 20ul real-time reaction. All reactions were duplicated. The method is very robust and we find that duplicate reactions for each sample are sufficient and accurate enough to obtain values for piRNA expression levels. Briefly, 10ul 2× TaqMan Universal Master Mix (ABI), 0.1ul 200uM Forward Primer, 0.2ul 100uM Universal Reverse Primer, and 0.2ul 20uM TaqMan Probe were used for each real-time PCR reaction. The PCR condition is: 95°C for 10min, followed by 40 cycles at

95°C for 15sec, and 60°C for 1min. All assays were done as duplicate. All the reactions were performed on ABI Prism 7000 Sequence Detection System.

RESULTS

A real-time PCR-based piRNA expression assay was designed (Figure 1). It is a barcoded multiplexed analysis of piRNA. Firstly a multiplexed Reverse Transcription (RT) step to convert multiple piRNAs into corresponding cDNAs by a set of stem-loop-structured reverse primers with barcode sequences, which contains eight nucleotides at its 3' end complementary to a specific piRNA, a stem sequence of barcode nucleotides unique for each piRNA, and a loop sequence common to all reverse primers. Then a multiplexed pre-amplification step was done to amplify these piRNA cDNA unbiased by PCR by a universal reverse primer and a set of each piRNA specific forward primers, which contain 3' end sequence that corresponds to the 5' end of each piRNA and 5' end sequence barcoded for every piRNA to increase the specificity and T_m to over 65°C. Finally a set of single plex real time PCR reactions were run to assess the amount of individual piRNA cDNAs. The universal reverse primer is used for both multiplexed pre-amplification PCR and single-plex real time PCR assays. It consists of the loop sequence of the reverse primers with an extra sequence added to its 5' end to increase T_m. Each real time PCR probe has a 5' FAM dye label (F) and a 3' minor groove binder with nonfluorescent quenching activity (MGB). The sequences of primers and probes are listed in the Table 1.

First, to test the specificity of the method, we run the multiplex piRNA assay using ten different tissues (Figure 2). We found that all eight piRNAs of Mili-binding or Miwi-binding class express specifically in testis, but not in ovary, brain, heart, muscle, liver, kidney, stomach, spleen, and lung, which exactly match the Northern blot result in Girard's and Aravin's papers (See Figure 1 of Girard et al and Figure 2 of Aravin et al) (4, 5). This proved that our multiplex piRNA assay specifically detect piRNA expression and no any background signal from other tissues.

Then we want to ask how sensitive of our method. We serially dilute the total RNA of testis and run the multiplex piRNA assay for these eight piRNAs (Figure 3). We found that it can accurately measure the expression for all eight tested piRNAs covering five orders of dynamic range. And it definitely works to 10pg total RNA level, which is equivalent of the RNA amount in a single cell. The correlation coefficients were better than 0.98 except piRNA-5 (0.96) for 5-logs dynamic range (10 ng to 1 pg). The expecting slope for 10-fold titration curves is 3.33 in log scale. The curve fitting had slopes range from 3.0 – 3.6 except piRNA-5 (with slop = 4.3). The large deviation of piRNA-5 was due to the data point of 10 pg had Ct value 40 or not detected. So our multiplex piRNA assay can quantitatively detect at least eight piRNAs expression simultaneously from as little as total RNA of a single cell.

Then with this new method, we want to know if piRNA also express in primordial germ cells (PGCs) before birth. PiRNA binding protein, Mili start to express in PGCs at Embryonic day 12.5 (E12.5). So we choose ten different stages from E12.5 stage until adult stage and check piRNA expression in male genital ridges and testis (Figure 4). We found that for Mili-binding piRNAs (piRNA-Chr9 and piRNA-Chr17; Figure 4F, G), they start to express in male germ cells (Pachytene Spermatocyte stage) of 14 days after birth (P14) testis and arrive at highest level in P21 testis and sustain that to adult stage. Mili-binding piRNAs does not express at detectable level in primordial germ cells from E12.5 to neonatal stages (E12.5, E14.5, E16.5, E18.5, P1 and P7).

For the Miwi-interacting piRNAs (piR-1, piR2, piR3, piR4, piR5), they start to express in male germ cells of P14 testis at significant level and achieve highest expression level at P21

in testis and maintain to adult stage (Figure 4A-E). Miwi-interacting piRNAs cannot be detected in PGCs from E12.5 to neonatal stages. For piRNA-T4, the pattern is similar (Figure 4H).

So both Mili-interacting and Miwi-interacting piRNAs start to express in male germ cells in P14 testis when the germ cells develop to Pachytene Spermatocyte stage. Before that, piRNAs are not expressed in primordial germ cells or neonatal testis even when Mili protein already expressed in PGCs.

DISCUSSION

PiRNAs are emerging as a third class of key small non-coding RNAs after siRNAs and microRNAs. It is abundantly expressed in male germ cells during meiosis. Its crucial function for spermatogenesis just starts to be explored, such as repressing transposon's activity to keep the integrity of the genome in germ line. The synthesis and processing of piRNAs are also under intensive investigation. It is heavily needed a sensitive and reliable method other than Northern blot to detect piRNAs during biochemical analysis for piRNA processing or piRNA complex activity. Here we reported a barcode primer directed real-time PCR-based multiplex assay for piRNAs, which can easily detect piRNA expression accurately at 0.01ng level amount of total RNAs. It can be used for biochemical purification activity of piRNA processing complex or other similar purposes. It can also be used for check piRNA expression dynamics during spermatogenesis. Because we integrate barcode primers into this assay, it is possible to pool multiple barcoded primers for microRNAs and piRNAs together to simultaneously detect both microRNAs and piRNAs from a single cell. Our assay has the potential to accelerate the process of dissect piRNA related mechanisms.

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REFERENCES

1. Carthew RW. Molecular biology. A new RNA dimension to genome control. *Science*. Jul 21; 2006 313(5785):305–6. [PubMed: 16857925]
2. Lin H. piRNAs in the germ line. *Science*. Apr 20.2007 316(5823):397. [PubMed: 17446387]
3. Hartig JV, Tomari Y, Forstemann K. piRNAs--the ancient hunters of genome invaders. *Genes Dev*. Jul 15; 2007 21(14):1707–13. [PubMed: 17639076]
4. Lau NC, Seto AG, Kim J, Kuramochi-Miyagawa S, Nakano T, Bartel DP, Kingston RE. Characterization of the piRNA complex from rat testes. *Science*. Jul 21; 2006 313(5785):363–7. Epub 2006 Jun 15. [PubMed: 16778019]
5. Aravin A, Gaidatzis D, Pfeffer S, Lagos-Quintana M, Landgraf P, Iovino N, Morris P, Brownstein MJ, Kuramochi-Miyagawa S, Nakano T, Chien M, Russo JJ, Ju J, Sheridan R, Sander C, Zavolan M, Tuschl T. A novel class of small RNAs bind to MILI protein in mouse testes. *Nature*. Jul 13; 2006 442(7099):203–7. [PubMed: 16751777]
6. Girard A, Sachidanandam R, Hannon GJ, Carmell MA. A germline-specific class of small RNAs binds mammalian Piwi proteins. *Nature*. Jul 13; 2006 442(7099):199–202. [PubMed: 16751776]
7. Grivna ST, Beyret E, Wang Z, Lin H. A novel class of small RNAs in mouse spermatogenic cells. *Genes Dev*. Jul 1; 2006 20(13):1709–14. [PubMed: 16766680]
8. Ro S, Park C, Song R, Nguyen D, Jin J, Sanders KM, McCarrey JR, Yan W. Cloning and expression profiling of testis-expressed piRNA-like RNAs. *RNA*. Aug 13.2007 [Epub ahead of print].
9. Kuramochi-Miyagawa S, Kimura T, Ijiri TW, Isobe T, Asada N, Fujita Y, Ikawa M, Iwai N, Okabe M, Deng W, Lin H, Matsuda Y, Nakano T. Mili, a mammalian member of piwi family gene, is essential for spermatogenesis. *Development*. Feb; 2004 131(4):839–49. [PubMed: 14736746]

10. Carmell MA, Girard A, van de Kant HJ, Bourc'his D, Bestor TH, de Rooij DG, Hannon GJ. MIWI2 is essential for spermatogenesis and repression of transposons in the mouse male germline. *Dev Cell*. Apr; 2007 12(4):503–14. [PubMed: 17395546]
11. Deng W, Lin H. miwi, a murine homolog of piwi, encodes a cytoplasmic protein essential for spermatogenesis. *Dev Cell*. Jun; 2002 2(6):819–30. [PubMed: 12062093]
12. Grivna ST, Pyhtila B, Lin H. MIWI associates with translational machinery and PIWI-interacting RNAs (piRNAs) in regulating spermatogenesis. *Proc Natl Acad Sci U S A*. Sep 5; 2006 103(36):13415–20. Epub 2006 Aug 24. [PubMed: 16938833]
13. Aravin AA, Sachidanandam R, Girard A, Fejes-Toth K, Hannon GJ. Developmentally regulated piRNA clusters implicate MILI in transposon control. *Science*. May 4; 2007 316(5825):744–7. [PubMed: 17446352]
14. Tang F, Hajkova P, Barton SC, Lao K, Surani MA. MicroRNA expression profiling of single whole embryonic stem cells. *Nucleic Acids Res*. Jan 24.2006 34(2):e9. [PubMed: 16434699]

Figure 1.

Schematic figure of the multiplex piRNA expression assay. Using piR-1 as an example. The letters with green shading corresponds to piR-1 sequence. The letters with red shading corresponds to barcode sequence in reverse primer and TaqMan probe. The letters with yellow shading corresponds to barcode sequence in forward primer.

Figure 2.

Specificity of the multiplex piRNA assay. (A-H) Expression of piR-1, piR-2, piR-3, piR-4, piR-5, piRNA-Chr17, piRNA-Chr9, piRNA-T4 in Heart, Liver, Lung, Kidney, Brain, Stomach, Muscle, Spleen, Ovary, and Testis of adult mice. Standard deviation (SD) is plotted as error bar in the figures. All 8 piRNAs were detected in testis from 10 pg RNAs. But, they were not detected after 40 cycles of PCR in other tissues. We also increase RNAs input up to 10 ng and confirmed that piRNAs were only expression in testis, not in other tissues (data not show).

Figure 3.

Sensitivity and accuracy of the multiplex piRNA assay. (A-H) Step-wise ten-fold dilution of purified total RNA from adult testis, from 100ng to 1pg for piR-1, piR-2, piR-3, piR-4, piR-5, piRNA-Chr17, piRNA-Chr9, piRNA-T4. Standard deviation (SD) is plotted as error bar in the figures. The titration curves of each piRNA assay for testis samples were fitted with linear equations for Ct values and total RNA amounts in pg. The different interceptions indicate the difference of assay efficiency in the RT and PCR reactions. As we discussed in our previous microRNA assays (12), to have high specificity in RT (reverse transcriptase) reactions, we designed our reverse primers (RP) with only 8 bases matched to the 3' ends of piRNAs. The T_m and the priming efficiency will have large variations. To obtain the

absolute quantity of each piRNAs, a standard curve using synthetic templates or a known quantity of samples, such as the titration curves of testis in Fig. 2, is needed. Since the slopes are similar, the relative amount or delta Ct values can be used to calculate the relative abundance of each piRNAs in different samples, which is similar to the fluorescent intensity ratios of a typical microarray analysis.

Figure 4.

Expression dynamics of piRNAs in PGCs and testis. (A-H) Expression assay of piRNAs in PGCs and testis from E12.5 stage to adult stage for piR-1, piR-2, piR-3, piR-4, piR-5, piRNA-Chr17, piRNA-Chr9, piRNA-T4. Standard deviation (SD) is plotted as error bar in the figures. NTC means 'No Template Control'.

Table 1

Primers and Probes for multiplex piRNA assay. The reverse primers are pooled together for multiplex reverse transcription reaction. The forward primers and the universal reverse primer are for both pre-amplification step and real-time PCR quantification step. TaqMan probes are for real-time PCR reactions.

order	piRNA Name	piRNA ID	piRNA Sequence (5'- 3')	Reverse Primer (5'- 3')	Forward Primer (5' - 3')	TaqMan Probe (5' - 3')
1	piR-1	DQ539889	UGACAUGAACACAGGUGGUCUCAGAUAGCUUU	TGATGCAGGTGTCGTGGAGTCGGCAAATTTCTGCATCAAAAAGCTAT	GCAGGGTGAACA CAGGTGCTCAGAT	(6-FAM)TTCCTGCATCAAAA GCTAT(MGB)
2	piR-2	DQ539890	UGUGUGAUGCAUACGACGGUGUCUGCGGU	ATGATGAAGTGTCGTGGAGTCGGCAAATTTTCATCATAC CAGCAG	CCAGGGGATGCATACGACGGTGCT	(6-FAM)TTTTTCATCATACCAGCAG(MGB)
3	piR-3	DQ539891	UGAGAGUGGCAUCUAAAUGUUUAGUGGU	CCAAATTGAGTGTCTGTGGAGTCGGCAAATTTCAAATTTGGACCACTAA	GGTGGGAGTGGCATCTAAAATGTTT	(6-FAM)TTTCAATTGGACCACTAA(MGB)
4	piR-4	DQ539892	UGACAUGAACACAGGUGGUCUCAAUAGCUUU	TTGATGATGTCTGTGGAGTCGGCAAATTATCATCAAAAAGCTAT	GCAGGGTGAACA CAGGTGCTCAAAAT	(6-FAM)TTATCATCAAAAAGCTAT(MGB)
5	piR-5	DQ539893	AAAAAAAACACAAAGGAUUUGAUUUGCCAAG	GATGAAGGTTGTCGTGGAGTCGGCAAATTCCTTTCATCCTTTGGCAA	GTCCAGGGA AAAACACAAGGATTTGATT	(6-FAM)TTCCTTTCATCCTTTGGCAA(MGB)
6	piRNA -Chr9	DQ692867	UUCUAGGUCUAGUAUUUUCUCCUAGGGA	ACTGGTCCGTGTCGTGGAGTCGGCAAATTGGACCAGTTCCTTAGG	GCCGTTGGGTAGGTCTAGTATTTTCTCC	(6-FAM)TTGGACCAGTTCCCTAGG(MGB)
7	piRNA -Chr17	DQ701619	UAAAA GACGAGUGAGAACUAA CAA GGA	TCA TGACTGTGTCGTGGAGTCGGCAAATTAGTCATGATCCTTGT	GGCTGGGAAA GACGAGTGAGAACTAA	(6-FAM)TTAGTCATGATCCTTGT(MGB)
8	piRNA -T4	DQ687473	UAGACAGCUUAGGACACUGAAAAAUUGUCUA	TGATTGATGTCTGTGGAGTCGGCAAATTATCAATCATAGACAAT	GCCGAGGGAGCTTAGGACACTGAAAAAT	(6-FAM)TTATCAATCATAGACAAT(MGB)