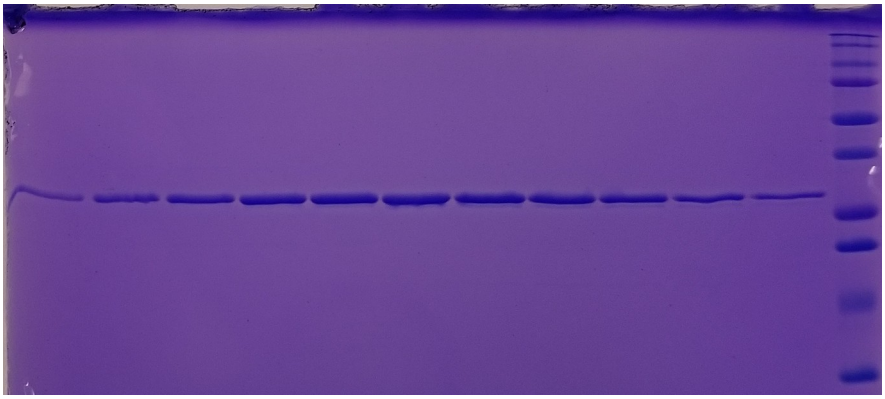
Figure legends:

Fig.S1. The fractionation of His-tag-purified PIR-1 using FPLC. The recombinant PIR-1 enriched with His-tag purification was further fractionated using FPLC, and the resulting fractions were visualized using a 12% denaturing protein PAGE gel.

Fig.S2. The analyses of mRNA-seq. A) the distribution of reads: each gene is divided into 100 fractions with mapped reads represented by the first nt position; the 'Y' axis represents the combined reads from all the genes mapped to each fraction; the 'X' axis represents each fraction of all the genes. **B) the comparison of gene expression:** each dot represents a gene with 'X' mapped reads in the sample prepared using nuclease P1 and 'Y' mapped reads prepared using alkali hydrolysis.

Fig.S1



34 35 36 37 38 39 40 41 42 43 44

FPLC fractions of purified His_6-PIR-1

Fig.S2

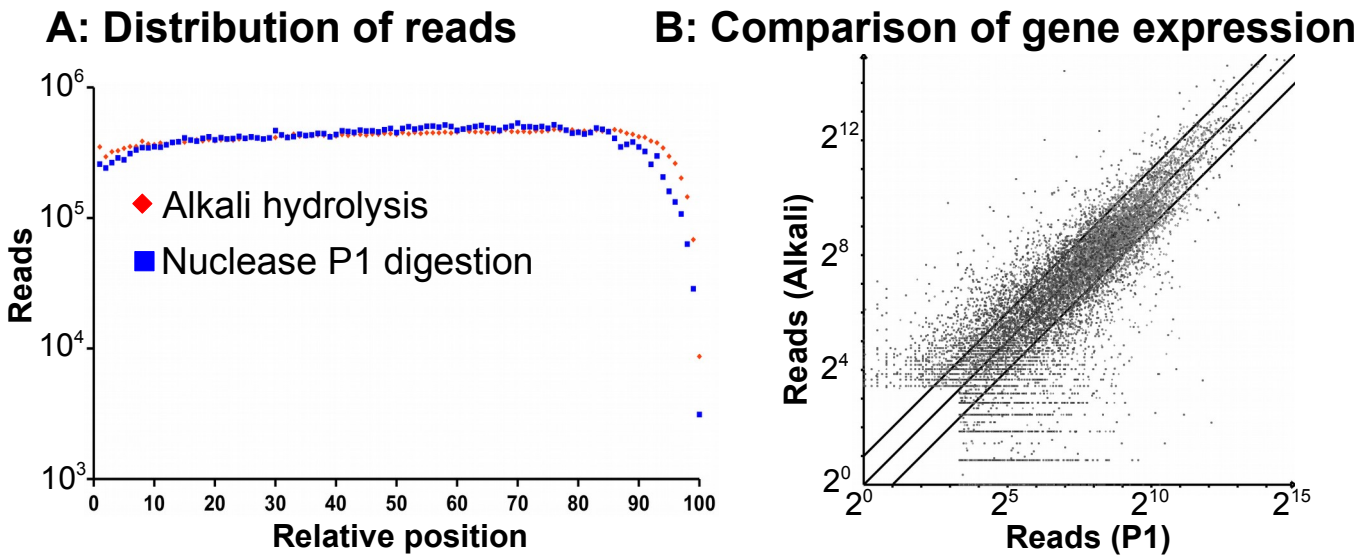

A: Distribution of reads
B: Comparison of gene expression
Reads
Relative position
Alkali hydrolysis
Nuclease P1 digestion
Reads (Alkali)
Reads (P1)

Table S1: Linkers and Primers

3' linker:	AppAGATCGGAAGAGCACACGTCTGAACTCCAGTCA/dideoxyC/
5' linker:	ACACUCUUUCCCUACACGACGCUCUUCCGAUCU
RT primer:	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
5' PCR primer:	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGA
3' PCR primer:	CAAGCAGAAGACGGCATACGAGAT ATCACGCA GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT CGATGTCT GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT TTAGGCGT GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT TGACCACC GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT ACAGTGCG GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT GCCAATTT GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT CAGATCGG GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT ACTTGATG GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT GATCAGTT GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT TAGCTTTT GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT GGCTACGG GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT CTTGTAAT GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT AGTCAAGT GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT AGTTCCAC GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT CCGTCCCA GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT GTAGAGCA GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT GTCCGCTC GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT GTGAAACT GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT GTGGCCGG GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT GTTTCGCC GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT CGTACGTA GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT GAGTGGCG GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT GGTAGCTA GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT ATGAGCGA GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT CAAAAGGC GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT CAACTACC GTGACTGGAGTTCAGACGTGT
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	CAAGCAGAAGACGGCATACGAGAT CACTCATA GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT CATTTTCG GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT CCAACAGC GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT CGGAATTC GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT CTATACTC GTGACTGGAGTTCAGACGTGT
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	CAAGCAGAAGACGGCATACGAGAT TCATTCTG GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT TCCCGAAC GTGACTGGAGTTCAGACGTGT
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	CAAGCAGAAGACGGCATACGAGAT TCGGCATG GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT CTTCGGCC GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT TTGTTACA GTGACTGGAGTTCAGACGTGT
	CAAGCAGAAGACGGCATACGAGAT AATCCAAT GTGACTGGAGTTCAGACGTGT

CAAGCAGAAGACGGCATACGAGAT	TCCTTTAA	GTGACTGGAGTTCAGACGTGT
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CAAGCAGAAGACGGCATACGAGAT	CCTGACTC	GTGACTGGAGTTCAGACGTGT
CAAGCAGAAGACGGCATACGAGAT	TAGCACAC	GTGACTGGAGTTCAGACGTGT
CAAGCAGAAGACGGCATACGAGAT	TCGGCGGG	GTGACTGGAGTTCAGACGTGT
CAAGCAGAAGACGGCATACGAGAT	TCAATTTC	GTGACTGGAGTTCAGACGTGT
CAAGCAGAAGACGGCATACGAGAT	GTAGGGAA	GTGACTGGAGTTCAGACGTGT
CAAGCAGAAGACGGCATACGAGAT	AAGGGTGG	GTGACTGGAGTTCAGACGTGT
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CAAGCAGAAGACGGCATACGAGAT	TGGAGGGG	GTGACTGGAGTTCAGACGTGT
CAAGCAGAAGACGGCATACGAGAT	TCAGGAAG	GTGACTGGAGTTCAGACGTGT
CAAGCAGAAGACGGCATACGAGAT	ACCACAAC	GTGACTGGAGTTCAGACGTGT
CAAGCAGAAGACGGCATACGAGAT	TTCAAGCC	GTGACTGGAGTTCAGACGTGT
CAAGCAGAAGACGGCATACGAGAT	TACTCAGA	GTGACTGGAGTTCAGACGTGT
CAAGCAGAAGACGGCATACGAGAT	TTCCTACT	GTGACTGGAGTTCAGACGTGT
CAAGCAGAAGACGGCATACGAGAT	TTATATGG	GTGACTGGAGTTCAGACGTGT
CAAGCAGAAGACGGCATACGAGAT	CCGTGACA	GTGACTGGAGTTCAGACGTGT
CAAGCAGAAGACGGCATACGAGAT	TAGCGCCA	GTGACTGGAGTTCAGACGTGT
CAAGCAGAAGACGGCATACGAGAT	CCTGGAGC	GTGACTGGAGTTCAGACGTGT
CAAGCAGAAGACGGCATACGAGAT	CCCGCAAT	GTGACTGGAGTTCAGACGTGT

RNA in red; all oligos are written from 5' to 3'; blue are barcodes

Table S2: A simplified working protocol for RNA cloning

Note:

A. Each reaction is carried out in a single PCR tube with multiple steps by adding components sequentially to carry out different functionalities.

B. The final concentration of each component at an individual step is based on the accumulative (total) volume (number in red on line 1 of each table), including the volume of the previous step and the volume of the added components (starting from line 2 of each table).

Step 1: 3' ligation (0.1-1 µg total RNA or 0.1 µg small RNA <200nt)

	stock conc.	final conc.	10 µl/Rx	4 Rxs
H2O/small RNA			3.25 µl	13 µl
Ligation buffer without ATP	10 X	1 X	1 µl	4 µl
PEG-8000	50%	25%	5 µl	20 µl
3' linker	20 µM	0.5 µM	0.25 µl	1 µl
Truncated T4 RNA ligase 2	20 µM	0.5 µM	0.25 µl	1 µl
PIR-1 (optional)	10 µM	0.25 µM	0.25 µl	1 µl

Mix thoroughly by pipetting 20 times and incubate at room temperature for 2 hrs

The volume of each reaction is 10 µl.

Step 2: annealing

Inactivate at 65 °C for 10 mins, add 0.5 µl of 10 µM RT primer and anneal at 65 °C for 5 mins

Cool to room temperature by 0.1 °C/s

The volume of each reaction is 10.5 µl due to the addition of the RT primer.

Step 3: 5' ligation

	stock conc.	final conc.	20 µl/Rx	4 Rxs
H2O			8.1 µl	32.4 µl
ATP	20 mM	0.5 mM	0.5 µl	2 µl
5' linker	20 µM	0.4 µM	0.4 µl	1.6 µl
T4 RNA ligase 1	20 µM	0.25 µM	0.25 µl	1 µl
hDCP2 (optional)	20 µM	0.25 µM	0.25 µl	1 µl

Add 9.5 µl of the mixture to each reaction, and incubate at room temp. for 2 hrs

The volume of each reaction is 20 µl including 10.5 µl from the previous step.

Step 4: Reverse transcription (RT)

	stock conc.	final conc.	24.25 µl/Rx	4 Rxs
dNTP	10 mM	0.41 mM	0.99 µl	3.98 µl
RT dilution buffer	12 X	1 X	2.02 µl	8.08 µl
DTT	100 mM	4 mM	0.97 µl	3.88 µl
SSII	20 µM	0.21 µM	0.25 µl	1.02 µl

Add 4.25 µl of the mixture to each reaction, and incubate at 42 °C 30 mins & 85 °C 5mins

The volume of each reaction is 24.25 µl including 20 µl from the previous step.

Step 5: PCR

	stock	final	50 µl/Rx	4 Rxs
H2O			37.25 µl	149 µl
PFU buffer	10 X	1 X	5 µl	20 µl
TMAC	1000 mM	15 mM	0.75 µl	3 µl
dNTP	10 mM	0.1 mM	0.5 µl	2 µl

5' PCR primer	10 μM	0.1 μM	**0.5** μl	**2** μl
Indexed 3' PCR primer	10 μM	0.1 μM	**0.5** μl	**2** μl
The above cDNA	10 X	1 X	**5** μl	**20** μl
PFU	100 X	1 X	**0.5** μl	**2** μl

1 cycle **94 °C 1min**
5 cycles **94 °C 20s; 53 °C 20s; 68 °C 40s**
11 cycles **94 °C 20s; 68 °C 40s**
4 °C forever

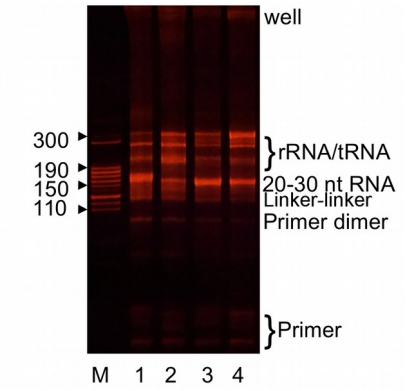
The volume of each reaction is 50 μl.

Add 3 μl each of 5' and indexed 3' PCR primer of 10 μM

2 cycles **94 °C 20s; 68 °C 40s**
4 °C forever

The volume of each reaction is 56 μl including the additional primers.

Indexed PCR products were compared visually using a 8% native PAGE gel, pooled according to a custom-specified ratio, phenol-extracted, precipitated and gel-purified as one sample.



M: 10 bp marker (110-200, 260 and 300)
1: 0.5 μg mouse testis total RNA
2: 0.4 μg mouse ovary total RNA
3: 0.5 μg *C. elegans* small RNA (<200 nt)
4: 1 μg *C. elegans* total RNA

Buffers

10 X ligation buffer without ATP:
0.5 M Tris pH7.5, 0.1 M $MgCl_2$, 0.1 M DTT

12 X RT dilution buffer:
250 mM Tris pH 8.8, 0.75 M KCl

Enzymes:
T4 RNA ligase 1 ((homemade)
Truncated T4 RNA ligase 2 (homemade)
Superscript II (SSII) from Invitrogen or homemade
PFU (commercial or homemade)
PIR-1 (homemade)
hDCP2 (homemade)
TMAC (Tetramethylammonium chloride, Sigma)

The theoretical size of a cDNA containing a 22-nt RNA insert is 146 bps. However, the apparent size on a PAGE gel may appear as 140-160 bps due to the sequence heterogeneity.