

Video Article

Single Read and Paired End mRNA-Seq Illumina Libraries from 10 Nanograms Total RNA

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Abstract

Whole transcriptome sequencing by mRNA-Seq is now used extensively to perform global gene expression, mutation, allele-specific expression and other genome-wide analyses. mRNA-Seq even opens the gate for gene expression analysis of non-sequenced genomes. mRNA-Seq offers high sensitivity, a large dynamic range and allows measurement of transcript copy numbers in a sample. Illumina's genome analyzer performs sequencing of a large number ($> 10^7$) of relatively short sequence reads (< 150 bp). The "paired end" approach, wherein a single long read is sequenced at both its ends, allows for tracking alternate splice junctions, insertions and deletions, and is useful for *de novo* transcriptome assembly.

One of the major challenges faced by researchers is a limited amount of starting material. For example, in experiments where cells are harvested by laser micro-dissection, available starting total RNA may measure in nanograms. Preparation of mRNA-Seq libraries from such samples have been described^{1,2} but involves significant PCR amplification that may introduce bias. Other RNA-Seq library construction procedures with minimal PCR amplification have been published^{3,4} but require microgram amounts of starting total RNA.

Here we describe a protocol for the Illumina Genome Analyzer II platform for mRNA-Seq sequencing for library preparation that avoids significant PCR amplification and requires only 10 nanograms of total RNA. While this protocol has been described previously and validated for single-end sequencing⁵, where it was shown to produce directional libraries without introducing significant amplification bias, here we validate it further for use as a paired end protocol. We selectively amplify polyadenylated messenger RNAs from starting total RNA using the T7 based Eberwine linear amplification method, coined "T7LA" (T7 linear amplification). The amplified poly-A mRNAs are fragmented, reverse transcribed and adapter ligated to produce the final sequencing library. For both single read and paired end runs, sequences are mapped to the human transcriptome⁶ and normalized so that data from multiple runs can be compared. We report the gene expression measurement in units of transcripts per million (TPM), which is a superior measure to RPKM when comparing samples⁷.

Video Link

The video component of this article can be found at <http://www.jove.com/video/3340/>

Protocol

1. Isolate total RNA

1. Add 1ml Trizol to cells/tissue, and homogenize with 18-22 gauge needle if necessary.
2. Add 200 μ l chloroform, and spin at 14,000 rpm for 30 minutes at 4 °C.
3. Take out top aqueous layer, add 0.5 μ l linear acrylamide, then add 500 μ l isopropanol. Let sit at room temperature for 20 minutes.
4. Spin at 14,000 rpm at 4 °C, wash pellet with 70% ethanol, and vacuum dry.
5. Resuspend in 1 μ l nuclease free water and transfer to 200 μ l PCR tube.

2. Prepare double stranded cDNA

1. To the above 1 μ l RNA add 1 μ l of 100 μ M oligo-dT-T7 reverse transcription primer (5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGCGGTTTTTTTTTTTTTTTTTTTTTTT-3'), heat at 70 °C (heat block) for 5 min, and snap cool on ice.
2. Add 1 μ l 5x FS buffer, 0.5 μ l DTT, 0.5 μ l dNTP mix and 0.5 μ l RnaseOUT (from SuperScript II kit).
3. Heat to 42 °C in PCR machine for 1 minute, then add 0.5 μ l Superscript II reverse transcriptase. Incubate for 1 hr at 42 °C.

4. Heat at 70 °C for 10 minutes, cool to 4 °C.
5. On ice, add the following to the above reaction:
 - Nuclease free water - 22.75 µl
 - 5X Second strand buffer - 7.5 µl
 - dNTP mix - 0.75 µl
 - E. coli DNA Ligase - 0.25 µl
 - E. coli DNA polymerase - 1 µl
 - RnaseH - 0.25 µl

Incubate for 2 hr at 16 °C then add 1 µl T4 DNA Polymerase, and incubate for additional 10 minutes at 16 °C.

6. Transfer to 1.5 ml eppendorf tube, and add 80 µl water.
7. Add 0.5 µl of linear acrylamide.
8. Add 72 µl 3 M NH₄OAc and 480 µl of cold 100% ethanol. Precipitate for 1 hr at -20 °C.
9. Spin at 14,000 rpm at 4 °C for 30 minutes, wash with 1 mL 70% ethanol, centrifuge at 14,000 rpm for 2 minutes, and vacuum dry for about 10 minutes.

3. Amplify poly-A mRNA by *in vitro* transcription

1. Resuspend above cDNA in 3.5 µl nuclease free water.
2. To the above, add 1 µl each of the dNTPs (total 4 µl), 1 µl of 10X reaction buffer and 1 µl of T7 polymerase, and 0.5 µl of RnaseOUT from Megascript kit.
3. Incubate at 37 °C in PCR machine overnight.
4. Ready for next step. Can be stored at -80 °C.

4. Fragmentation of amplified poly-A mRNA

1. Add 26 µl of water to above reaction and 4 µl 10x fragmentation reagent.
2. Heat in PCR machine at 70 °C for exactly 7 minutes.
3. Add 5 µl of fragmentation stop buffer, put sample on ice.

5. RNA clean up

1. To the above reaction, add 60 µl water and 350 µl of buffer RLT from Rneasy MinElute kit, and mix by pipetting.
2. Add 250 µl ethanol, mix by pipetting, and pipette into spin column.
3. Spin at 8000 rcf for 20 seconds.
4. Wash once with RPE, spin for 20 seconds, wash second time with 80% EtOH, spin for 2 minutes, and dry column with 5 minute spin.
5. Elute RNA in 10 µl nuclease free water.

6. cDNA synthesis

First strand synthesis for single read library:

1. In a PCR tube, add the following:
 - Fragmented Poly-A mRNA - 10 µl
 - NotI Random nonamer Primer - 1 µl

Incubate at 70 °C for 5 minutes in thermocycler, and quick chill on ice. NotI Nonamer Primer (5'-TGAATTCGCGGCCGCTCAAGCAGAAGACGGCATACGAGCTCTCCGATCT NNNNNNNNN -3'). The 5' proximal sequence is the NotI restriction site while the next sequence until the random region is the reverse complement of Illumina's adaptor B sequence from Chip-Seq kit.

2. To the above, add the following on ice (from SuperScript III kit):
 - 5X first strand buffer -4 µl
 - DTT - 2 µl
 - dNTP mixture - 1.5 µl

Put on thermocycler for 2 minutes at 42°C, add 1 µl of SuperScript III reverse transcriptase, and incubate at 42 °C for 1hr.

*Instead of dCTP, 5-methyl dCTP was used in the dNTP mixture.

First strand synthesis for paired end library:

1. In a PCR tube, add the following:
 - Fragmented Poly-A mRNA - 10 µl

- Random hexamer primer - 1 μ l

Incubate at 65 °C for 5 minutes in thermocycler, and quick chill on ice.

2. To the above, add the following on ice (from SuperScript firststrand III kit):

- 5X first strand buffer - 4 μ l
- DTT - 2 μ l
- dNTPs (from kit) - 1.5 μ l

Put on thermocycler for 1 min at 45 °C, add 1 μ l of SuperScript III reverse transcriptase, and incubate at 45 °C for 1 hour.

Second strand synthesis (for both libraries):

3. To the above, add the following on ice (from SuperScript II kit):

- Water (RNase free) - 91 μ l
- 5X Second Strand Buffer - 30 μ l
- dNTPs (from kit) - 3 μ l
- E. coli DNA Ligase - 1 μ l
- E. coli DNA polymerase - 4 μ l
- E. coli RNase H - 1 μ l

Mix tube by inversion, give a short spin and incubate at 16 °C for 2 hr.

7. Purify cDNA

1. Purify cDNA sample with Zymo columns, elute in 40 μ l of water for single read and 30 μ l of water for paired end library.

8. End repair

For single read library:

(Use Illumina Chip-Seq sample prep kit)

1. To the above 40 μ l of cDNA, add:
 - T4 DNA ligase buffer with 10mM ATP - 5 μ l
 - dNTP mix - 2 μ l
 - T4 DNA polymerase - 1 μ l
 - Klenow enzyme (diluted 1:5 with water to 1U/ μ l) - 1 μ l
 - T4 PNK - 1 μ l

Incubate in the thermal cycler for 30 minutes at 20 °C.

For paired end library:

(Use Illumina paired end sample prep kit)

1. To the above 30 μ l cDNA, add:
 - RNase DNase free water - 45 μ l
 - T4 DNA ligase buffer with 10mM ATP - 10 μ l
 - 10mM dNTP mix - 4 μ l
 - T4 DNA polymerase - 5 μ l
 - Klenow enzyme - 1 μ l
 - T4 PNK - 5 μ l

Incubate in the thermal cycler for 30 minutes at 20 °C.

9. cDNA clean up

1. Clean up cDNA using Zymo columns. Elute in 34 μ l of EB for single read and 32 μ l of EB for paired end library.

10. Add 'A' bases to the 3' end of the DNA fragments

For single read library:

1. Prepare the following reaction mix:
 - DNA sample - 34 μ l

- Klenow buffer - 5 μ l
- dATP - 10 μ l
- Klenow exo - 1 μ l

Incubate for 30 minutes at 37 °C.

For paired end library:

1. Prepare the following reaction mix:

- DNA sample - 32 μ l
- Klenow buffer - 5 μ l
- dATP - 10 μ l
- Klenow exo - 3 μ l

Incubate for 30 minutes at 37 °C.

11. cDNA clean up

1. Clean up cDNA using zymo columns. Elute in 10 μ l of EB.

12. Adapter ligation

For single read library:

(Use Illumina Chip-Seq sample prep kit)

1. Prepare the following reaction mix:

- DNA sample - 10 μ l
- Adapter Oligo Mix - 1 μ l
- 2X DNA Ligase Buffer - 15 μ l
- DNA ligase - 4 μ l

Incubate for 15 minutes at room temperature. Adapters should be thawed on ice and diluted 1:20.

For paired end library:

(Use Illumina paired end sample prep kit)

1. Prepare the following reaction mix:

- DNA sample - 10 μ l
- PE Adapter Oligo Mix - 10 μ l
- 2X DNA Ligase Buffer - 25 μ l
- DNA ligase - 5 μ l

Incubate for 15 minutes at 20 °C. Adapters should be thawed on ice and diluted 1:20.

13. Ligation reaction clean up

1. Clean up the ligation reaction using zymo columns. Elute in 44 μ l of water for single read library and 6 μ l of EB followed by another elution in 5 μ l of EB for paired end library.

Perform a NotI digestion, ONLY for single read library

- cDNA - 44 μ l
- NEB buffer 3 - 5 μ l
- BSA - 0.5 μ l
- NotI - 1 μ l

Incubate for 2 hr to overnight at 37 °C, and purify reaction using zymo column. Elute with 6 μ l of buffer EB followed by a second elution with 5 μ l of EB.

14. Size selection/Gel purification

Perform the following using a 2% Sybr Safe E-Gel from Invitrogen.

1. Run Program 0-PreRun 2 minutes.
2. Load 1kb plus ladder from Invitrogen diluted 1:4, and load 10 μ l.

3. Load all of DNA sample, and fill empty lanes with 10 μ l of water.
4. Run Program 1-EGel 2% Run 28 minutes.
5. Size select 200-300 bp gel slice with a fresh razor blade.

15. Elute DNA from gel slice

1. Weigh gel slice, and add 3 volumes of buffer QG to 1 volume of gel (use Gel Extraction kit from Qiagen)
2. Incubate at 50 °C for 10 minutes or until gel slice dissolves.
3. Add 1 volume of isopropanol, and mix by inversion or pipetting.
4. Add to column, spin for 1 minute at maximum speed, and discard flow-through.
5. Add 500 μ l of Buffer QG to column, spin for 1 minute at maximum speed, and discard flow through.
6. Add 750 μ l of Buffer PE to column, spin for 1 minute at maximum speed, and discard flow through.
7. Centrifuge for 1 minute at maximum speed.
8. Elute in 36 μ l of EB for single read and 23 μ l EB for paired end library.

16. PCR

For single read library:

(Use Illumina Chip-Seq sample prep kit)

1. Prepare the following PCR reaction mix:
 - DNA - 36 μ l
 - 5X Phusion buffer - 10 μ l
 - dNTP mix - 1.5 μ l
 - PCR primer 1.1 - 1 μ l
 - PCR primer 2.1 - 1 μ l
 - Phusion polymerase - 0.5 μ l

Use the following PCR protocol:

- 30 seconds at 98 °C
- 10 cycles of:
 - 10 seconds at 98 °C
 - 30 seconds at 65 °C
 - 30 seconds at 72 °C
- 5 minutes at 72 °C
- Hold at 4°C

*The first time that the kit is used, dilute PCR primers 1:2 with EB buffer.

For paired end library:

(Use Illumina paired end sample prep kit)

1. Prepare the following PCR reaction:
 - DNA - 23 μ l
 - Phusion DNA polymerase - 25 μ l
 - PCR primer PE 1.1 - 1 μ l
 - PCR primer PE 2.1 - 1 μ l

Amplify using the following PCR protocol:

1. 30 seconds at 98°C
2. 10 cycles of:
 - 10 seconds at 98°C
 - 30 seconds at 65°C
 - 30 seconds at 72°C
3. 5 minutes at 72°C
4. Hold at 4°C

*The first time that the kit is used, dilute PCR primers 1:2 with EB buffer.

17. Library clean up

1. Clean up library using zymo columns. Elute in 12 μ l of EB

18. Quantitate library

1. Quantitate library using Qubit. It is ready for sequencing. Use sequencing primers from Illumina single read cluster generation kit V4 or higher for single read library and Illumina paired end cluster generation kit V4 or higher for paired end library.

19. Data analysis

Bowtie⁶ was used to map reads to the RefSeq gene set (NCBI Build 36.1). The single end reads (30 nucleotides) and the pair end reads (42 nucleotides) were mapped allowing up to 10 matches to the gene set, and allowing up to two mismatches per read. Transcripts Per Million (TPM) values were obtained to measure gene expression using RSEM⁷ (RNA-Seq by Expectation-Maximization).

20. Representative Results: We made T7LA libraries for both single read and paired end runs from 1 μ g, 100 μ g, 10 μ g, 1 μ g and 100 μ g starting total RNA (Figure 1). For evaluation of our protocol, we made single read and paired end libraries without T7 RNA amplification starting from 10 μ g total RNA. These control libraries, termed "MinAmp", have minimal amplification. The only amplification they undergo are the 10 cycles of PCR near the end of the protocol to ligate the Illumina sequencing adapters, a step common to all libraries. All RNA used were isolated from H14 human embryonic stem cells⁸.

We first evaluated the number of genes identified by the various libraries (Table 1 and Supplementary Table 1). For both single read and paired end libraries, the 10 ng T7LA libraries identified almost the same number of genes as the 10 μ g MinAmp libraries, with a TPM of 10 or more. In the case of the single read libraries, the 10 ng T7LA library identified 100% of the 8500 genes identified by the 10 μ g unamplified library. For paired end libraries, the 10 ng T7LA library identified 86% of genes identified by the 10 μ g unamplified library (7961 of 9267 genes). Libraries made from less than 10 ng were not able to identify as many genes. For example, in the single read protocol, the 1 ng library identified only ~50% of the genes identified by the 10 μ g MinAmp library, prompting us to limit the lowest amount of total RNA for use with the T7LA protocol to 10 ng. Moreover, mapping of a housekeeping gene, GAPDH (Figure 2) shows that all T7LA libraries made with at least 10ng of starting RNA identified all exons, including the extreme 5' exon. Comparison of the 10 ng T7LA single read and paired end libraries with the MinAmp libraries shows a high degree of similarity (Spearman correlation, $R = 0.90$ and 0.95 respectively, Figures 3a and b). We also compared the two single read and paired end libraries made from 10 ng total RNA and they had a very high correlation coefficient ($R = 0.92$), demonstrating that both types of libraries made using the T7LA protocol produce a very similar gene expression signature (Figure 3c.). Hence, the T7LA method is able to produce sequencing libraries that are as reliable and comprehensive as the MinAmp libraries, but from 1000-fold less starting material.

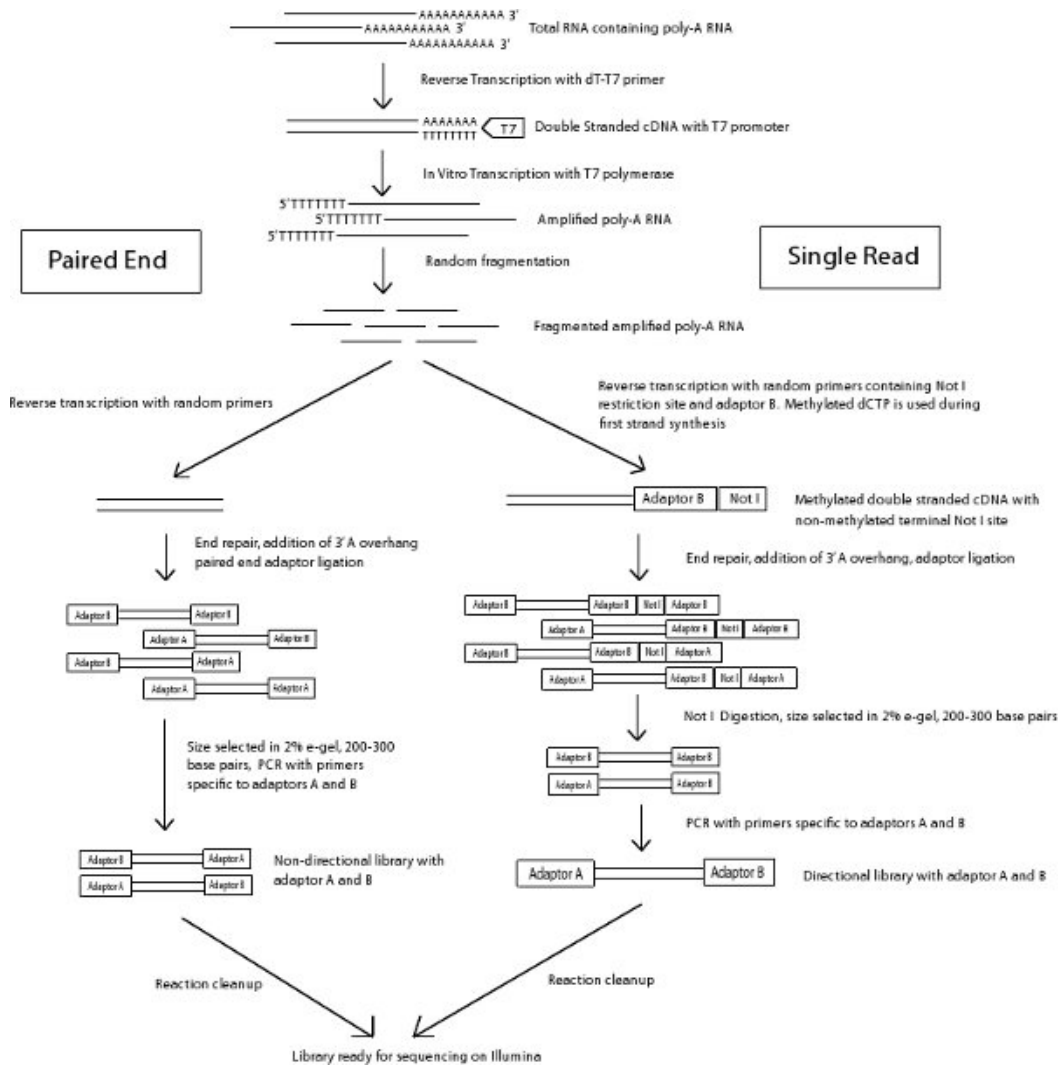
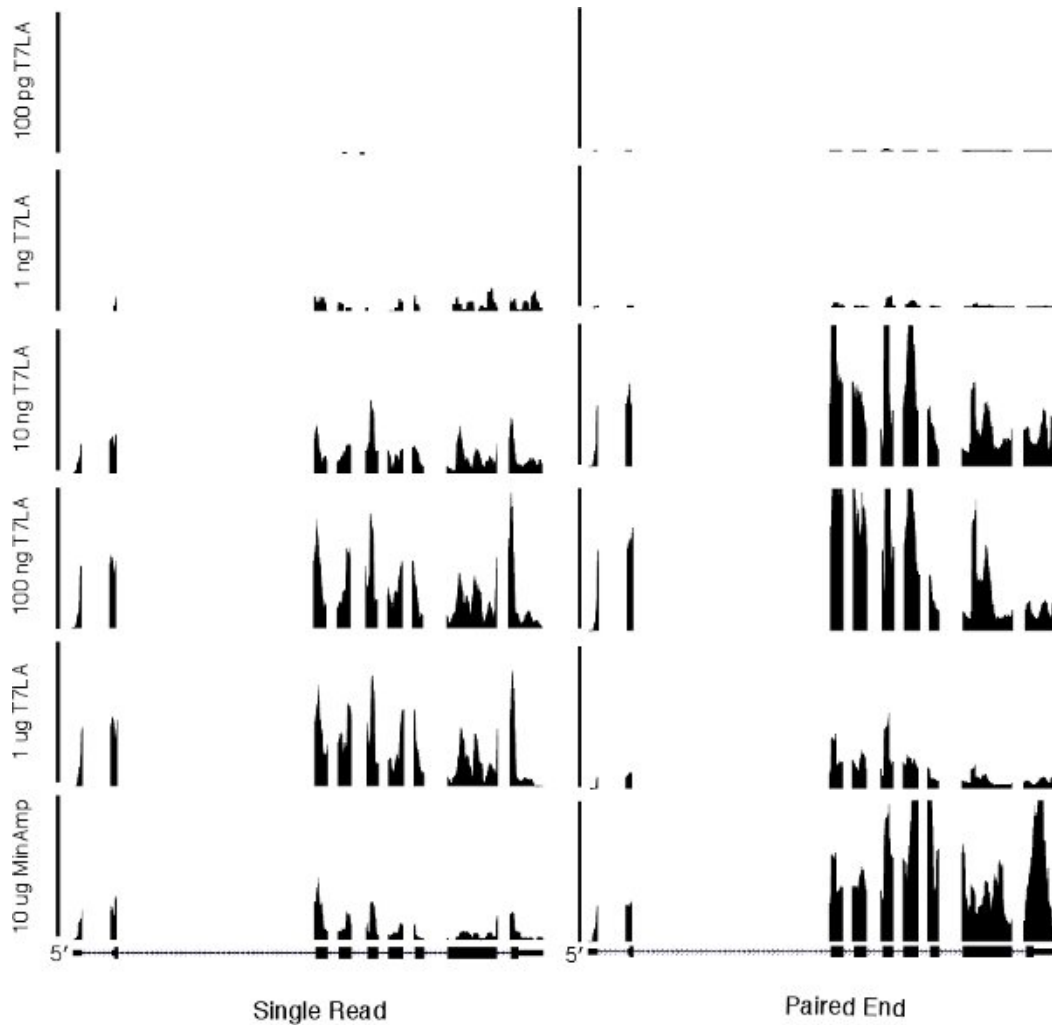


Figure 1 Schema of paired end and single read library preparation protocol.



GAPDH

Figure 2 A genome browser picture of a housekeeping gene, GAPDH, for all single read and paired end libraries. The scale bar on the left for the single read libraries indicates 350 total reads. The scale bar in the center for the paired end libraries indicates 5000 total reads. The horizontal axis represents the genome sequence of GAPDH.

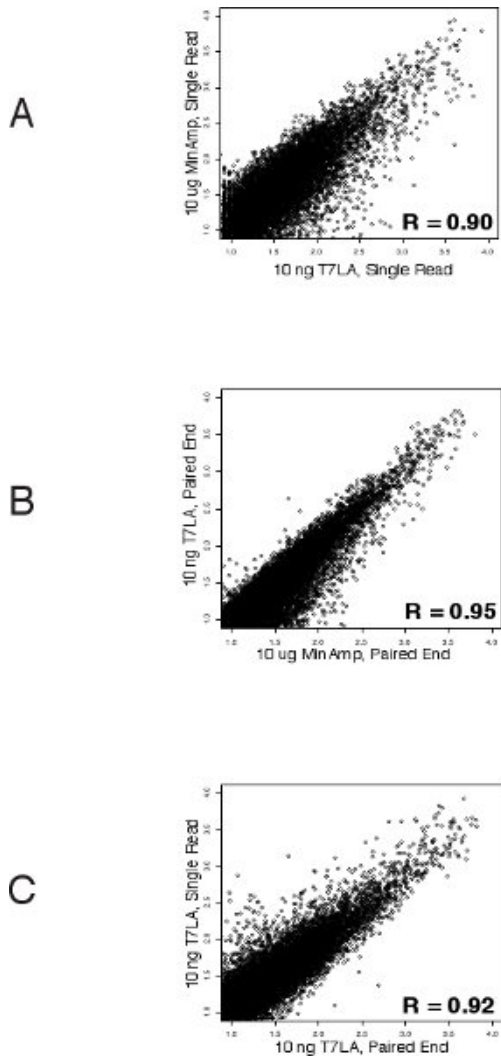


Figure 3 Correlation of gene expressions between the various libraries (Spearman's): **A.** Between single read 10 ng T7LA and 10 μ g MinAmp library shows that both these libraries have a very similar gene expression pattern ($R = 0.90$). **B.** Between paired end 10 ng T7LA and 10 μ g MinAmp library demonstrates their similarity of gene expression profiles ($R = 0.95$). **C.** Correlation of gene expressions between 10 ng paired end and 10 ng single read libraries show a high degree of similarity between these libraries prepared by the T7LA method ($R = 0.92$).

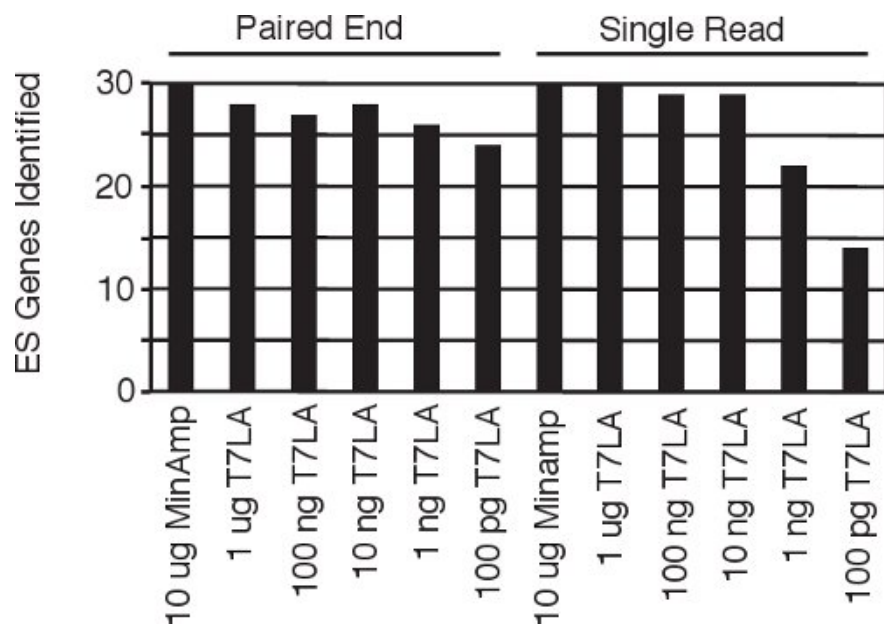


Figure 4 Identification of human embryonic stem cell specific genes5 from all the single read and paired end libraries.

Library	Type°	Raw Clusters	% Clusters passing filter	% Aligning to genome	% Error Rate	Genes identified
MinAmp 10ug	Single read	225602 +/- 4952	65.48 +/- 2.58	47.61 +/- 0.53	0.62 +/- 0.06	8500
1ug T7LA	Single read	144818 +/- 6513	82.21 +/- 6.45	48.09 +/- 0.27	0.42 +/- 0.03	8757
100ng T7LA	Single read	27385 +/- 1818	81.33 +/- 11.75	44.46 +/- 4.53	0.49 +/- 0.10	8709
10ng T7LA	Single read	11184 +/- 985	60.70 +/- 3.70	14.96 +/- 1.15	0.99 +/- 0.30	8589
1ng T7LA	Single read	12695 +/- 1365	53.27 +/- 16.76	4.08 +/- 0.79	2.25 +/- 1.56	4720
100pg T7LA	Single read	10390 +/- 1398	72.99 +/- 2.90	1.48 +/- 0.20	1.51 +/- 0.39	1121
MinAmp 10ug	Paired End R1	95786 +/- 12937	90.77 +/- 2.79	58.50 +/- 0.95	0.94 +/- 0.38	9267
	Paired End R2	95786 +/- 12937	90.77 +/- 2.79	58.13 +/- 1.13	0.99 +/- 0.37	
1ug T7LA	Paired End R1	297669 +/- 10196	91.35 +/- 0.36	46.89 +/- 0.14	0.47 +/- 0.01	7334
	Paired End R2	297669 +/- 10196	91.35 +/- 0.36	45.52 +/- 0.12	0.51 +/- 0.01	
100ng T7LA	Paired End R1	205602 +/- 9932	90.53 +/- 0.76	63.44 +/- 1.00	0.48 +/- 0.02	8011
	Paired End R2	205602 +/- 9932	90.53 +/- 0.76	61.80 +/- 8.09	0.60 +/- 0.36	
10ng T7LA	Paired End R1	214622 +/- 11155	89.98 +/- 1.13	56.32 +/- 1.94	0.80 +/- 0.26	7961
	Paired End R2	214622 +/- 11155	89.98 +/- 1.13	46.41 +/- 18.39	2.48 +/- 2.68	;
1ng T7LA	Paired End R1	144951 +/- 19841	90.54 +/- 1.19	3.91 +/- 0.16	8.71 +/- 0.86	8124
	Paired End R2	144951 +/- 19841	90.54 +/- 1.19	3.27 +/- 1.21	9.11 +/- 3.52	
100pg T7LA	Paired End R1	187600 +/- 11759	89.52 +/- 1.11	1.78 +/- 0.05	13.42 +/- 0.50	6623
	Paired End R2	187600 +/- 11759	89.52 +/- 1.11	1.99 +/- 0.23	15.29 +/- 0.96	

° R1 and R2 are forward and reverse sequences of a tag

* ≥10 TPM

Table 1. Information on cluster numbers, genes identified, error rate, percent alignment of the single read and paired end libraries.

Supplementary Table 1. List of all genes and their TPM values for all samples, single read and paired end.

Discussion

Current protocols for making paired end libraries require between 1 µg⁹ to 2.5 µg¹⁰ starting amount of total RNA. Here we present our linear T7 amplification based (T7LA) method to prepare both single read and paired end Illumina sequencing libraries and show that this method allows generation of libraries from as low as 10 ng of total RNA, producing data that is comparable to that of minimally amplified (MinAmp) libraries

made from 1000 fold more starting material (10 µg total RNA). The 10 ng libraries not only identify similar total numbers of genes, but also produce gene expression signatures that are similar (Figures 3a and b). Moreover, both the single read and paired end libraries produced by the T7LA method are very similar to each other (Figure 3c), which allows researchers to compare data generated by libraries made from either protocol. Since these libraries were prepared from human embryonic stem cell RNA, we searched for 30 stem cell specific genes among the libraries and find that almost all of these genes (93-100%) are identified by the libraries made from at least 10 ng of starting total RNA (Figure 4), thus validating our protocol. We believe our protocol would be very useful for researchers, especially in circumstances such as flow sorted cells or laser-micro-dissected tissue where starting material is limiting. In such circumstances, our protocol would allow generation of gene expression data comparable to libraries made from much larger starting quantities since our protocol produces expression profiles comparable across at least 3 orders of magnitude of starting RNA.

Disclosures

The authors disclose that J.A.T. is a founder, stockowner, consultant and board member of Cellular Dynamics International (CDI). He also serves as scientific advisor to and has financial interests in Tactics II Stem Cell Ventures.

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