Chapter 4

Application of RNA-Seq Technology in Cancer Chemoprevention

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Abstract

RNA-sequencing is a revolutionary tool to follow differential expression after treatment with cancer chemopreventive agents. It allows a real genome-wide screening independent of prior assumptions and is well suited for analyzing coding but also long noncoding RNAs. It still consents the discovery of new genes and isoforms and increased our knowledge of antisense and other noncoding RNAs in a tremendous manner. Moreover, it permits to detect low-abundance and biologically critical isoforms and reveals genetic variants and gene fusions in one single assay. Here, we provide a detailed protocol for stranded RNA-sequencing.

Key words RNA-Seq, Deep-sequencing, Chemoprevention, Transcriptome profiling, Long noncoding RNA, Stranded RNA-Seq, Transcriptional signature, Novel transcript and isoform discovery

1 Introduction

The development of RNA-sequencing (RNA-Seq)has allowed many advances in the genome-wide transcriptional profiling [1]. RNA-Seq is based on next generation sequencing and consents the quantification of all transcripts longer than 200 nucleotides, including those that are not annotated yet, and therefore still gives rise to the discovery of new genes and transcripts. It revolutionized our understanding of the complexity of transcription. There is no limit of the dynamic range of transcript detection due to the technique, permitting therefore also the detection of rare RNA transcripts and of more differentially expressed genes with a higher fold change [2]. Furthermore, it allows the identification of alternative splicing, allelic-specific expression, and posttranscriptional RNA editing events. But also gene fusions or genetic variants can be reliably detected.

Despite the classical RNA-Seq, there are also several alternative methodologies described in literature. RNA immunoprecipitation sequencing (RIP-Seq) allows mapping RNA-protein interactions [3]. Argonaute HITS-CLIP decodes microRNA-mRNA interac-

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tion maps [4]. Ribo-Seq identifies those mRNAs that are actively translated [5] whereas global run-on sequencing (GRO-Seq) is quantifying transcription by directly measuring nascent RNA production [6].

To perform a classical RNA-sequencing, there are two decisions to make. The first one involves the rRNA removal that can be done either by poly(A) purification of the RNA or by ribosomal depletion. The latter retains the whole spectrum of RNA transcripts and therefore also transcripts that do not contain a poly(A) tail. The second decision to take is whether the RNA-Seq should be stranded or not. A stranded RNA-Seq allows us to know immediately from which DNA strand a given RNA transcript is deriving from which is helpful for newly discovered transcripts but really indispensable for antisense transcript detection. Additionally it is thought to enhance alignment and transcript annotation.

Here, we describe the protocol for a stranded RNA-sequencing with previous ribosomal depletion, starting from the RNA extraction to the library preparation and the final setting up of the sequencing reaction including all necessary quality control and quantification steps (Fig. 1).

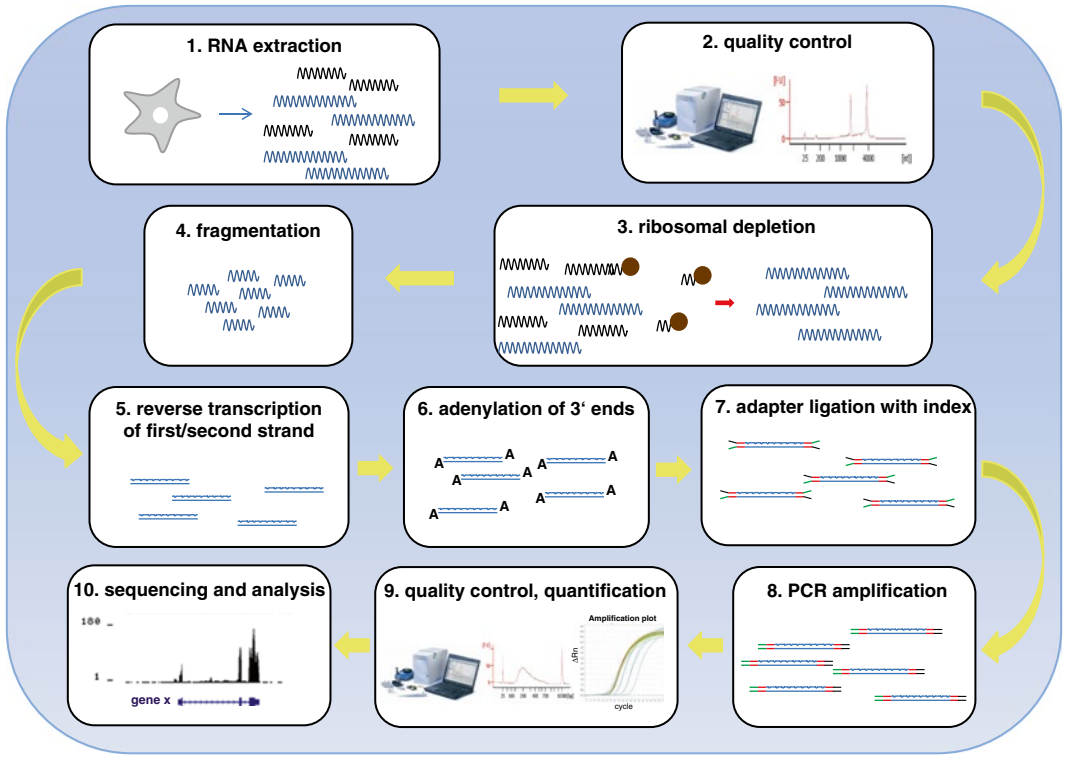

Fig. 1 Schematic overview of the library preparation steps for stranded RNA-sequencing

2 Materials

2.1 RNA-Extraction

1. miRNeasy Kit (Qiagen, Valencia, CA, USA).
2. RNase Zapper (Life Technologies, Carlsbad, CA, USA).
3. RNase-free ultrapure water.
4. Phosphate-buffered saline (PBS): To obtain a 10× stock solution add 80 g NaCl, 2 g KCL, 14.4 g Na_2HPO_4 (dibasic anhydrous), and 2.4 g KH_2PO_4 (monobasic anhydrous), fill up to 1 L with ultrapure water, and autoclave. The pH should be 7.4. The final 1× working concentration is 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4.
5. Ethanol (100 %).
6. QIAzol Lysis Reagent and RPE Buffer make part of the miRNeasy Kit (Qiagen, Valencia, CA, USA).
7. Chloroform.
8. RNase-Free DNase Set (cat. no. 79254, Qiagen, Valencia, CA, USA).
9. NanoDrop® spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).
10. Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA).
11. Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

2.2 Library Preparation and Quality Control

1. TruSeq Stranded Total RNA LT Sample Prep Kit with Ribo-Zero Gold (Illumina Inc., San Diego, CA, USA; part # RS-122-2301).
2. Magnetic stand-96 (Life Technologies, Carlsbad, CA, USA; part # AM10027).
3. Agencourt RNAClean XP 40 mL (Beckman Coulter Genomics, Fullerton, CA, USA).
4. Agencourt AMPure XP 60 mL (Beckman Coulter Genomics, Fullerton, CA, USA).
5. Agilent High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA).
6. SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA).
7. 80 % Ethanol: Prepare always fresh by adding 2 mL of ultrapure water to 8 mL 100 % ethanol.
8. 1 M Tris–HCl pH 8.5: Weigh 60.57 g Tris [Tris (hydroxymethyl) aminomethane] into 450 mL of ultrapure water. Adjust the pH with HCl, make up to 500 mL with ultrapure water, and filter through a 0.2 μM cellulose acetate filter.

9. 10 mM Tris–HCl pH 8.5 with 0.1 % Tween 20: Dilute 1 mL of 1 M Tris–HCl pH 8.5 into 50 mL of ultrapure water and add 100µL of Tween 20. Finally, the solution has to be filled up with ultrapure water to 100 mL.
10. Agilent DNA 1000 Kit or Agilent High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA).
11. Real-time PCR instrument like Applied Biosystems StepOne™.
12. KAPA SYBR FAST ABI PRISM Readymix, part # KK4604 (Kapa Biosystems, Wilmington, MA, USA).
13. qPCR primer 1.1: 5′ AATGATACGGCGACCACCGAGAT 3′ HPLC purified; qPCR primer 2.1: 5′ CAAGCAGAAGAC GGCATACGA 3′ HPLC purified.
14. Optional: KAPA Library Quantification Kit (Kapa Biosystems, Wilmington, MA, USA) (*see* **Note 21**).
15. cBot or Cluster Station (Illumina Inc., San Diego, CA, USA).
16. Genome Analyzer, NextSeq or HiSeq (Illumina Inc., San Diego, CA, USA).

3 Methods

3.1 RNA-Extraction

To extract sufficient material to perform an RNA-Seq experiment we usually start with 60 mm dishes containing cells being ca. 80 % confluent (*see* **Notes 1** and **2**). Perform the experiments in triplicate.

1. You can lyse the cells directly on the plate after two washes with ice-cold PBS by adding 700 µL of QIAzol Lysis Reagents (*see* **Note 3**). The solution is pipetted up and down onto the plate until the cells are getting lysed (*see* **Note 4**). Transfer the homogenate into a 1.5 mL tube and vortex for 1 min. Incubate at room temperature (RT) for 5 min. Add 140 µL of chloroform and shake vigorously for exactly 15 s. Incubate again at RT for 2–3 min. Centrifuge the tubes for 15 min at 12,000 × *g* at 4 °C (*see* **Note 5**).
2. Collect the upper aqueous phase and transfer it into a new tube. Add 1.5 volumes of ethanol 100 %, mix thoroughly (*see* **Note 6**), and transfer up to 750 µL into an RNeasy spin column. Centrifuge at room temperature for 30 s at 8000 × *g*. Remove and discard the flow-through. Repeat this step until all liquid was loaded onto the column.
3. Perform an on-column DNase digestion (*see* **Note 7**). The buffers and DNase enzymes are supplied in the RNase-free DNase Set. Wash the column with 350 µL buffer RWT by adding it on top of the column and centrifuge at 8000 × *g* for 30 s at RT. Discard the flow-through. Mix 10 µL of the DNase I

stock with 70 µL RDD and add all to the column. Incubate for 15 min at 20–30 °C. Subsequently wash again with 350 µL buffer RWT like described above.

4. Wash with 500 µL RPE buffer by adding it on top of the column and centrifuging at 8000 × *g* for 30 s at RT, and discard the flow-through. Repeat the washing with 500 µL RPE buffer and centrifuge for 2 min. Place the column in a new collection tube and centrifuge again for 1 min at 8000 × *g* to dry completely the column membrane. Place the column into a new 1.5 mL tube; add 30 µL RNase-free ultrapure water and centrifuge at 8000 × *g* for 1 min to elute the RNA.

3.2 RNA Quality Control

1. The quality of the extracted RNA should be evaluated to adjust later on the protocol for the library preparation. The quality can be determined using the Agilent RNA 6000 Nano Kit together with the Agilent Bioanalyzer. The quality of the RNA will be expressed as RIN (RNA integrity number) ranging from 1 to 10.
2. The RNA should be tested by qPCR with target genes that are known to be differentially expressed after chemopreventive treatment. Design the amplicons in the 50–150 bp range to be able to use them again after the library preparation.

3.3 Library Preparation

We usually start with 500 ng RNA for the library preparation (*see* **Note 8**). The steps to be performed are: removal of ribosomal RNA, fragmentation of the RNA, reverse transcription of the first strand with random hexamers, reverse transcription of the second strand, adenylation of the 3′end, ligation of Illumina adapters with indices, and finally PCR amplification (*see* **Note 9**).

3.4 Removal of Ribosomal RNA

1. Dilute 500 ng RNA in 10 µL ultrapure water in a 96-well 0.3 mL PCR plate (*see* **Note 10**) and add 5 µL of rRNA Binding Buffer. Add 5 µL of rRNA Removal Mix-Gold and mix well by pipetting up and down.
2. Seal the plate and denaturate the RNA in a thermal cycler for 5 min at 68 °C. Take the plate out of the thermal cycler and leave it at RT for 1 min.
3. Vortex the rRNA Removal Beads well and transfer 35 µL into each well of a *new* PCR plate. Add the denatured RNA mix by pipetting immediately quickly up and down (*see* **Note 11**) and incubate them for 1 min at RT. Place the plate on the magnetic stand and incubate for 1 min. Remove the supernatant and transfer it into a new 0.3 mL PCR plate. Control if no beads were carried over by placing the plate for 1 min on the magnetic stand.
4. Mix the RNAClean XP beads well by vortexing. Add 99 µL into each well of the 0.3 mL PCR plate containing ribosomal

depleted RNA and mix well by pipetting. If the RNA was degraded (below RIN 6–7), use instead 193 μL of beads. Incubate at RT for 15 min. Place the plate on the magnetic stand for 5 min and eliminate the supernatant. Add 200 μL of freshly prepared 70 % ethanol to the beads without disturbing them on the magnet, wait for 30 s and remove again the supernatant. Let the beads dry at RT for 15 min.

5. Remove the plate from the magnetic stand and elute the RNA from the beads by adding 11 μL of Elution Buffer and pipetting the beads up and down. Let them incubate for 2 min at RT and separate subsequently the RNA from the beads by placing the plate on the magnetic stand for 5 min. The supernatant contains the ribosomal depleted RNA.

3.5 RNA Fragmentation

1. Transfer 8.5 μL of the eluted ribosomal depleted RNA into a new 96-well 0.3 mL PCR plate and add 8.5 μL Elute, Prime, Fragment High Mix. Mix well by pipetting.
2. Seal the plate and fragment the RNA in a thermal cycler. Program setting: 94 °C for 8 min if the RNA was not degraded; hold at 4 °C (*see* **Note 12**). Briefly centrifuge the plate after the incubation.

3.6 Reverse Transcription of the First Strand

1. Transfer 50 μL of SuperScript II Reverse Transcriptase into the First Strand Synthesis Act D Mix and mix by pipetting (*see* **Notes 13** and **14**). Add 8 μL of it into each well of the 96-well PCR plate containing the fragmented ribosomal depleted RNA and mix by pipetting. Seal the plate with an adhesive seal and incubate the samples for the reverse transcription on the thermal cycler like following: 10 min 25 °C, 15 min 42 °C, 15 min 70 °C, hold at 4 °C. Perform immediately the second-strand synthesis.

3.7 Reverse Transcription of the Second Strand

The second cDNA strand will be generated by incorporating dUTP instead of dTTP. It will give rise to a double-stranded cDNA that is required for the subsequent Illumina Adapter ligation that permits PCR amplification, sequencing, and indexing. The dUTP ensures the strandedness because in the subsequent PCR amplification the polymerase will amplify only the strands that do not contain dUTP.

1. Add 5 μL of Resuspension Buffer to each well of the 96-well PCR plate. Add 20 μL Second Strand Marking Master Mix to each sample and mix by pipetting. Seal the plate with an adhesive seal and incubate for 1 h at 16 °C in a thermal cycler.
2. Purify the double stranded cDNA with 90 μL AMPure XP beads equilibrated to room temperature. Mix well and incubate for 15 min at RT. Place the plate on the magnetic stand for 5 min. Remove the supernatant and wash the beads still attached

to the magnet with 200 μL freshly prepared 80 % Ethanol. After 30-s incubation remove the supernatant and wash again with 200 μL 80 % ethanol like described before. Discard again the supernatant and let the beads air-dry at RT for 15 min. Remove the plate from the magnet and add 17.5 μL of Resuspension Buffer (equilibrated to RT) to the beads. Mix well by pipetting. Incubate for 2 min and subsequently place the plate on the magnet for 5 min. Transfer 15 μL of the supernatant into a new 96-well 0.3 mL PCR plate (*see* **Note 15**).

3.8 Adenylation of the 3′ End

In the subsequent step, the 3′ ends of the cDNA fragments will be adenylated. The attachment of a single A nucleotide will increase the efficiency to ligate the Illumina Adapters that contain a corresponding T'overhang. Furthermore, it will help avoiding a ligation between the cDNA fragments.

1. Add 2.5 μL of Resuspension Buffer and 12.5 μL of A-Tailing Mix to each well of the 0.3 mL PCR plate containing the cDNA. Mix well by pipetting, seal the plate with an adhesive seal and place the plate into a thermal cycler. Incubate for 30 min at 37 °C, followed by 5 min at 70 °C and a hold at 4 °C.
2. Proceed immediately to the adapter ligation.

3.9 Adapter Ligation

Illumina uses two color channels for sequencing, a red one for A/C and a green one for G/T. The adapters have to be chosen in a way that the samples that will be pooled and therefore sequenced together in one lane contain in each base position nucleotides from both channels. Please check the Illumina guidelines for pooling samples in the original "TruSeq Stranded Total RNA Sample Prep Guide."

1. Add 2.5 μL of Resuspension Buffer and 2.5 μL of Ligation Mix to each well of the adenylated cDNA and mix well by pipetting (*see* **Note 16**). Add 2.5 μL of the appropriate Adapter and mix well. Seal the plate and incubate in a thermal cycler for 10 min at 30 °C.
2. Add 5 μL of Stop Ligation Buffer to each well.
3. Purify the samples with AMPure XP Beads equilibrated to room temperature. Add 42 μL of beads to each sample and mix well by pipetting. Incubate at RT for 15 min. Separate the beads from the solution by incubating the plate on the magnet for 5 min. Discard the supernatant and wash the beads with 200 μL freshly prepared 80 % ethanol without disturbing the beads attached to the magnet. Wait for 30 s, remove the ethanol and wash again with 200 μL of 80 % ethanol. Air-dry the pellet for 15 min while still being attached to the magnet. Remove the plate from the magnet and resuspend the beads with 52.5 μL of Resuspension Buffer. After 2-min incubation

at RT place the plate on the magnet for 5 min. Transfer 50 μL of the supernatant into a new 0.3 mL PCR tube.

4. Purify again with AMPure XP Beads by adding 50 μL of beads to the samples. Mix well by pipetting and incubate for 15 min at RT. Place the plate on the magnetic stand and incubate for 5 min. Discard the supernatant and wash the beads by adding 200 μL of freshly prepared 80 % ethanol without disturbing the beads. Incubate for 30 s, remove the supernatant, and wash again with 200 μL of 80 % ethanol like described before. Remove completely the supernatant and allow the beads to air dry for 15 min while still being attached to the magnet. Remove the plate from the magnet and resuspend the beads with 22.5 μL of Resuspension Buffer. Incubate for 2 min and separate the beads from the supernatant by 5 min incubation of the plate on the magnetic stand. Transfer 20 μL of the supernatant into a new 0.3 mL PCR plate (*see* **Note 17**).

3.10 PCR Amplification

Here, all cDNA fragments will be amplified via the adapter attached before. The PCR amplification will enable you to perform the subsequent quality control steps but also ensure the enrichment of those cDNAs that have linkers on both sides. The adapters are necessary for (1) the binding to the flow cell, (2) the cluster formation, and (3) the sequencing reaction.

1. Add 5 μL of PCR primers and 25 μL of PCR master mix to each well of the PCR plate. Mix well by pipetting. Seal the plate and incubate in a thermal cycler at 98 °C for 30 s followed by 15 cycles of 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s and a subsequent elongation at 72 °C for 5 min and a hold at 4 °C (*see* **Note 18**).
2. Purify the PCR products with AMPure XP Beads. Centrifuge briefly the plate and add 50 μL of well dispersed beads slurry to each well of the PCR plate. Mix well by pipetting and incubate for 15 min. Separate the beads from the supernatant by placing the plate on the magnetic stand for 5 min. Discard the supernatant and wash the beads with 200 μL of freshly prepared 80 % Ethanol with the plate remaining on the magnet. Wait for 30 s and then remove the supernatant and wash again with 200 μL of 80 % Ethanol like described before. Eliminate all supernatant and allow the beads to air dry for 15 min. Remove the plate from the magnet and resuspend the beads in 32.5 μL of Resuspension Buffer. Incubate for 2 min and place subsequently the plate on the magnet for 5 min. Transfer 30 μL of the supernatant that contains the final library to a new 0.3 mL PCR plate.

3.11 Validation of the Library

The final library has to be well controlled before sequencing.

3.12 Quality Control

1. The quality will be verified with the Agilent Bioanalyzer using an Agilent DNA 1000 Kit or an Agilent High Sensitivity DNA Kit (*see* **Note 19**). The library should give a peak around 260–290 bp (Fig. 3a and *see* **Note 20**).
2. A second quality control could include a qPCR using the same primers that were employed before to validate the differential expression after chemopreventive treatment [7].

3.13 Quantification of the Library

The quantity will be assessed via qPCR using primers specific for the adapter regions (qPCR primer 1.1 and qPCR primer 2.1.).

1. Measure your library with a NanoDrop® spectrophotometer and dilute your library to ca. 5 ng/μL in Resuspension Buffer. Transfer 1 μL of this dilution into 500 μL of 10 mM Tris–HCl pH 8.5 with 0.1 % Tween 20.
2. Use a library with known concentration to prepare 2× serial dilutions for the standard. The standards should be in the range of 100, 50, 25, 12.5, and 6.25 pM. Dilute the DNA standard library in 200 μL 10 mM Tris–HCl pH 8.5 with 0.1 % Tween 20 (*see* **Note 21**).
3. Perform the qPCR in 20 μL reaction volume, using in each well of an optical 0.3 mL PCR plate 10 μL KAPA SYBR Fast Master Mix, 0.2 μL qPCR primer 1.1, 0.2 μL qPCR primer 2.1, 7.6 μL ultrapure water, and 2 μL of the standard or the new library. Conduct the qPCR at least in duplicate. Use the following thermal profile: 95 °C for 5 min, 40 cycles with 95 °C 3 s, 60 °C 30 s plus melting curve.

Calculate the concentration of your libraries with the following formula:

$$\frac{\text{quantity} \times \text{bp length of standard}}{\text{bp length of new library}} \times \text{dilution factor } 500 = \text{concentration (pM)}$$

3.14 Performing a Sequencing Run

The libraries have to be diluted to 10 nM and denatured with NaOH. Dilute the libraries in 10 mM Tris–HCl pH 8.5 to 10 nM (*see* **Note 22**). Add 2 μL of the 10 nM DNA library to 17 μL of 10 mM Tris–HCl pH 8.5 and denature for 5 min at RT with 1 μL 2 N NaOH. For setting up the instruments follow the manufacturer's instructions. The sequencing is divided into two steps. The first one comprises the binding of the DNA library to the flow cell and the cluster formation via bridge amplification which is the prerequisite

for the subsequent visualization of the sequencing reaction. The second step comprehends the sequencing itself.

3.15 Sequence Analysis

The sequence analysis comprises alignment and quality control of the reads, transcript assembly and quantification of the transcripts, and finally differential expression analysis. RNA-Seq is also well suited to analyze differential exon usage, gene fusions, and RNA editing. There are several commercial but also free RNA-Seq software packages available. TopHat and Cufflinks for instance are free, open-source software tools that perform the mapping, and transcript assembly and quantification, respectively. The differential expression can be analyzed via Cuffdiff [8, 9].

4 Notes

1. Take care that the cells are free of mycoplasms. The Ribominus protocol removes only ribosomal RNA and retains the whole spectrum of RNA molecules, including RNA from mycoplasms!
2. RNA is easily degraded by RNase enzymes. These enzymes are located within the cells but also on hands and labware. There are stable to heat and detergents. It is therefore essential to wear gloves and it is suggested to use RNA-zapper to decontaminate the pipettes and if necessary the work surfaces. Use RNase-free certified plasticware and ultrapure water. Use RNase-free barrier filter tips.
3. It is important to process cells immediately as soon as harvested to avoid changes in gene expression or RNA degradation. Solutions like QIAzol Lysis Reagents are phenol/guanidine based and function to lyse the cells but also contemporaneously to prevent RNA degradation due to its protein denaturing activity.
4. The solution is getting viscous.
5. The centrifugation results in the separation of an upper aqueous phase containing the RNA and an interphase and lower organic phase encompassing the DNA and proteins.
6. Do not centrifuge to avoid losing a precipitate that might have been formed.
7. It is highly recommended to remove all genomic DNA to avoid high background noise and the loss of strandedness in the RNA-sequencing (*see* Fig. 2).
8. The official protocol from Illumina recommends 0.1–1 μg RNA as starting material. Here, we describe the library preparation using the TruSeq Stranded Total RNA LT Sample Prep Kit from Illumina. But there are also stranded library prep kits from other

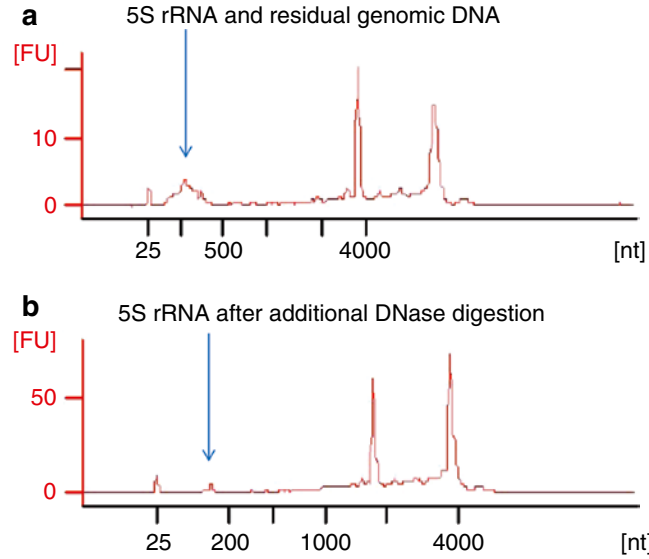

Fig. 2 Examples of human total RNAs on a Bioanalyzer electropherogram. (**a**) Total RNA without DNase treatment. (**b**) The same total RNA as in (**a**) after DNase digestion

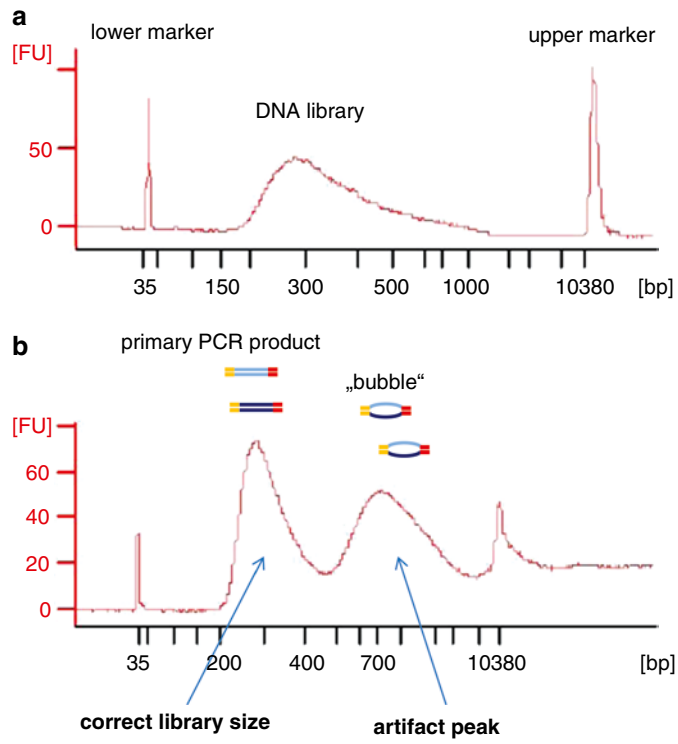

Fig. 3 Typical profiles from the Bioanalyzer representing stranded cDNA libraries. (**a**) Stranded cDNA library showing a peak in the expected size range. (**b**) Stranded cDNA library displaying a secondary artifact peak in the higher bp range

companies available like Epicentre (Illumina), Bio Scientific, Clontech, KAPA Biosystems, NuGen, Lexogenor, NEB.

9. Let all beads used in this protocol come to room temperature for 30 min before usage.
10. Later on the nucleic acids (RNA and after the reverse transcription DNA) will be purified by magnetic beads. The separation works best with 96-well PCR plates and a 96-well magnetic stand. The use of 1.5 mL microcentrifuge tubes with a corresponding magnet is possible but results sometimes in difficulties removing the supernatant from the beads. Therefore, it is recommended to use in each step directly the 96-well PCR plates, even with low-throughput samples.
11. Do not add the beads directly to the denatured RNA mix to ensure proper rRNA removal! Avoid foaming. The rRNA targeting oligos are biotinylated and can therefore be easily removed with streptavidin containing beads.
12. The time of incubation at 94 °C has to be modified if one is interested in increasing the insert size of the library or if the starting RNA material was degraded. Please consult the Appendix of the original Illumina protocol "TruSeq Stranded mRNA Sample Prep Guide."
13. Actinomycin D is toxic. It is included in the first strand buffer to inhibit DNA dependent DNA-synthesis that could occur after reverse transcription by using the newly generated cDNA strand as template again. As a consequence, the strandedness is improved and antisense artifacts due to spurious second-strand cDNA synthesis should be avoided [10].
14. To avoid multiple freeze and thaw cycles (not more than 6×) aliquot the first-strand synthesis mix into small aliquots. Immediately after use return them to −20 °C.
15. The protocol can be paused here. Store the plate at −20 °C.
16. Return the ligation mix immediately to −20 °C after use.
17. The protocol can be paused here. Store the plate at −20 °C.
18. The number of cycles might be decreased to reduce the possibility to introduce biases.
19. If using the Agilent High Sensitivity DNA Kit load around 5 ng onto a chip (the concentration can be determined by the NanoDrop® spectrophotometer).
20. If in the Bioanalyzer profile a second, bigger peak like in Fig. 3b is showing up, an over-amplification of the library occurred. The primers were used up and the different fragments hybridized with each other via the linker region forming a kind of bubble. You can still proceed to sequencing. Using the AMPure XP Beads for purification there should be no peak below 150 bp that could arise from primer dimers or adapter dimers.

21. If no DNA library with known concentration is available, one can use the "KAPA Library Quantification Kit" for the first library quantification which contains a set of standards.
22. Avoid repeated freeze and thaw of the 10 nM diluted library.

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