Published in final edited form as:
Methods Mol Biol. 2012 ; 883: 59–73. doi:10.1007/978-1-61779-839-9_4.

Strand-specific RNA-seq applied to malaria samples

Nadia Ponts, **Duk-Won D. Chung**, and **Karine G. Le Roch**
Institute for Integrative Genome Biology, Center for Disease Vector Research, Department of Cell Biology and Neuroscience, University of California, Riverside (CA) 92521, USA

Summary

Over the past few years only, next-generation sequencing technologies became accessible and many applications were rapidly derived, such as the development of RNA-seq, a technique that uses deep sequencing to profile whole transcriptomes. RNA-seq has the power to discover new transcripts and splicing variants, single nucleotide variations, fusion genes, and mRNA levels-based expression profiles. Preparing RNA-seq libraries can be delicate and usually obligates buying expensive kits that require large amounts of stating materials. The method presented here is flexible and cost-effective. Using this method, we prepared high quality strand-specific RNA-seq libraries from RNA extracted from the human malaria parasite *Plasmodium falciparum*. The libraries are compatible with Illumina®'s sequencers Genome Analyzer and Hi-Seq. The method can however be easily adapted to other platforms.

Keywords

strand-specific RNA-seq; high-throughput sequencing; malaria; *Plasmodium falciparum*; splicing variant discovery; transcript discovery

1. Introduction

The advent of high throughput sequencing technologies marked the beginning of a new era for whole genome analysis. The cost for sequencing a genome dropped considerably over the past five years, a revolution for labs focusing on genome mining. Applications were rapidly derived, applying deep sequencing to various “omics” such as the development of RNA-seq to analyze whole transcriptomes. Where microarray-based techniques proved to be powerful tools in exploring gene expression profiles, RNA-seq has the power to establishing expression profiles in a more quantitative manner and to discover new transcripts and splicing variants, single nucleotide variations, and fusion genes at the single-base resolution. The dark side of the application is the considerable amount of complex computations that accompany RNA-seq. In addition, where preparing gDNA libraries is robust and affordable, with a wide range of reagent options on the market, preparing RNA-seq libraries is more expensive and can be more challenging. Here we present a method that has the double advantage to use reagents originally designed for genomic DNA library preparation and to ultimately provide strand-specific information that simplifies downstream analysis. The described method is used to prepare, in a flexible and cost-effective manner, high quality

libraries from small amounts of RNA extracted from the human malaria parasite *Plasmodium falciparum* to be sequenced on Illumina®'s sequencers Genome Analyzer and Hi-Seq. It can however be adapted to a wide array of other organisms and platforms.

2. Materials

All materials and reagents must be molecular biology grade and nuclease-free. All solutions must be freshly prepared before each experiment. Lab benches and pipettes must be clean. The regular use of cleaning solutions such as RNase*Zap*® (Ambion) is recommended. Nuclease-free barrier tips should be used at all times. Always wear gloves and change them often. After tissue homogenization, samples should always be kept on ice. Using non-stick (low retention) RNase-free tubes and tips can be beneficiary when working with low amounts of RNA.

1. Parasite cultures grown in complete RPMI medium at 5 % hematocrit (*see* **Note 1**)
2. TRIzol® LS Reagent (Invitrogen™) pre-warmed at 37°C
3. Chloroform
4. Isopropanol pre-chilled on ice
5. Nuclease-free non-DEPC water
6. DNAse I RNAse-free (Ambion®)
7. Deionized formamide
8. Formaldehyde 37 %
9. 10X MOPS EDTA buffer pH 7.0
10. Glycerol 50 %
11. Bromophenol blue powder
12. Ethidium bromide 20 mg/mL
13. GenElute™ mRNA Miniprep Kit (Sigma-Aldrich)
14. 5X RNA storage solution (Ambion)
15. HPLC-purified random hexamers and Anchored Oligod$T_{(20)}$
16. SuperScript® VILO™ cDNA synthesis kit (Invitrogen™)
17. DNA Clean & Concentrator™ (Zymo Research)
18. 5X First Strand Buffer (Invitrogen™): 250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$

[1]Typically, parasites are cultured in 25 mL total volume at 5 % hematocrit until a parasitemia of 6-10 % is reached. If a synchronization is performed (*e.g.*, using sorbitol) make sure to let the parasites recover from the stress of the treatment, ideally wait for one cycle of invasion, before harvesting the RNAs. Waiting will minimize the background caused by stress-related variations. This RNA-seq protocol is typically prepared using 4 different cultures pooled together.

19. 5X Second Strand Buffer (Invitrogen™): 100 mM Tris-HCl (pH 6.9), 450 mM KCl, 23 mM $MgCl_2$, 0.75 mM β-NAD^+, 50 mM $(NH_4)_2SO_4$
20. Set of dATP, dGTP, dCTP, and dUTP
21. *E. coli* DNA Polymerase I 10 U/μL (Invitrogen™)
22. *E. coli* DNA Ligase 10 U/μL (Invitrogen™)
23. *E. coli* DNA RNase H 2 U/μL (Invitrogen™)
24. 0.1 M DTT
25. dsDNA Shearase™ (Zymo Research)
26. Encore™ NGS Library System I (NuGEN®)
27. Same-day 70 % ethanol in nuclease-free water
28. USER™ Enzyme (New England Biolabs®)
29. 1X TE buffer pH 8.0

3. Methods

3.1. Total RNA Extraction from Parasite Cultures

1. Spin down the cultures at 700 g for 5 min with brake level set at the minimum. Aspirate off the supernatant.
2. Add 5 volumes of pre-warmed TRIzol® LS (37°C) and mix thoroughly to dissolve all clumps (*see* **Note 2**).
3. Incubate at 37°C for 5 min to ensure the complete de-proteinization of nucleic acids.
4. *Stopping point:* the samples can be stored at -80°C until further processing. They must be thawed on ice before resuming the protocol.
5. Keep the samples on ice. For each 5 mL of TRIzol® LS that was used in step 2, add 1 mL of chloroform and vortex for 1 min.
6. Centrifuge at 12000 g for 30 min at 4°C.
7. Carefully transfer the upper aqueous layer to a fresh tube (*see* **Note 3**) and add 0.8 volume of pre-chilled isopropanol to precipitate the RNA. Mix carefully by inverting.
8. *Stopping point:* the tubes can be stored at -20°C overnight until further processing. Their temperature must be equilibrated on ice for a few minutes before resuming the protocol (*see* **Note 4**).

[2]It is crucial to dissolve everything at this step for an optimal yield.
[3]Do not transfer any of the lower phase to the next step. Phenol inhibits downstream enzymatic reactions including reverse transcription.
[4]Tubes containing nucleic acids that were precipitated at -20°C or -80°C should always be allowed to equilibrate on ice before centrifugation. At these low temperatures the samples tend to become very viscous and the efficiency of centrifugation is lower.

9. Mix by inverting and centrifuge at 12000 g for 30 min and 4°C. Carefully aspirate off the supernatant.
10. Allow the pellet to air-dry on ice for 5 min and add 30-100 μL of RNase-free non-DEPC treated water.
11. Heat tubes at 60°C for 10 min and then place on ice.

3.2. DNase Treatment

1. To 100 μL of RNA solution add 11.3 μL of 10X DNase I Buffer and 2 μL (4 U) of DNase I (2 U/μL).
2. Incubate the tube at 37°C for 30 min.
3. Inactivate the DNAse at room temperature for 5 min in the presence of 1 mM EDTA. Transfer the tube on ice.
4. *Stopping point:* RNA solutions can be aliquoted and stored at -80°C. When needed, thaw tubes on ice. Avoid repeated freeze/thaw cycles.

3.3. Verification of the quality and the quantity of total RNA

1. Quantify the concentration of the total RNA solution by UV spectrophotometry, such as a NanoDrop (Thermo Scientific). Typically, a clean solution of nucleic acid in nuclease-free water has an OD ~ 1.85. A ratio ranging from 1.8 to 2.2 is therefore recommended.
2. Check RNA integrity by agarose gel electrophoresis (*see* **Note 5** and **Note 6**).
3. If genomic DNA is visible on the gel repeat the DNase treatment (*see* **Note 7**).
4. Verify the absence of trace amounts of genomic DNA by 40 cycles of PCR on a chosen control gene using 50-500 ng of total RNA solution. Repeat the DNase treatment if necessary.
5. *Stopping point:* Store the total RNA solution at -80°C.

3.4. Purification of polyA+ mRNA from Total RNA

This protocol uses the reagents from the GenElute™ mRNA Miniprep Kit (Sigma-Aldrich). Before starting, equilibrate a heating block for microcentrifuge tubes at 70°C. Keep the Elution Solution at 70°C. If the beads were kept at 4°C let them sit on the bench top for at least 15 min. Cold beads reduce yields.

---

[5]Typically, 0.5 to 1 μg of total RNA should be loaded on a 1.2 % agarose gel. Mix sample with 10 volumes of denaturing RNA loading buffer (for 1.5 mL stock loading buffer mix: 750 μL of deionized formamide, 240 μL of formaldehyde 37 %, 150 μL of 10X MOPS EDTA buffer pH 7.0, 200 μL of 50 % glycerol, 0.5 mg of bromophenol blue, and 10 μL of ethidium bromide 10 mg/mL) and heat for 5 min at 65°C. Ensure that all solutions and hardware, including electrophoresis tank and gel combs, are RNAse-free. The 28S and 18S rRNAs should appear as two clean bands around 5.3 and 2 kb respectively. The upper band should be more intense. The presence of significant smearing or a lower intensity of the upper band indicates degradation of the extracted material.

[6]If the purity of the RNA solution is questioned, *e.g.*, presence of phenol, the samples can be further purified on RNeasy® (QIAGEN) clean-up columns according to the “RNA Cleanup” manufacturer's protocol. All solutions must be fresh.

[7]It is crucial to eliminate all contamination with genomic DNA in order to avoid competition in downstream reaction and inaccurate quantitative analysis of RNA levels or false discovery of alternative transcripts.

1. Thaw the total RNA sample on ice. The amount of starting material should be 150 to 500 μg of purified total RNA. The remaining steps are performed at room temperature unless specified otherwise.
2. Adjust volume of total RNA to 250 μL with RNase-free water. Add 250 μL of 2X Binding Solution and vortex briefly.
3. Add 15 μL of Oligo(dT) Polystyrene Beads and vortex thoroughly.
4. Heat the mixture at 70°C for 3 min to denature the RNA and let it cool down for 10 min at room temperature.
5. Centrifuge 2 min at maximum speed (14000-16000 g) in a tabletop microcentrifuge. Carefully pipette off the supernatant without disturbing the bead pellet (*see* **Note 8**).
6. Add 500 μL of Wash Solution mix by vortexing. Transfer the mixture to a GenElute spin filter/collection tube assembly. Failure to transfer all traces of mixtures will result in lower mRNA yields.
7. Centrifuge 1 min at maximum speed (14000-16000 g) in a tabletop microcentrifuge. Discard the flow-through and place the collection tube back on the GenElute spin filter.
8. Add 500 μL of Wash Solution onto the GenElute spin filter and centrifuge 2 min at maximum speed. Transfer the GenElute spin filter to a fresh nuclease-free microcentrifuge tube.
9. Add 50 μL of Elution Solution heated at 70°C onto the center of the GenElute spin filter and incubate 5 min at 70 °C. Centrifuge 1 min at maximum speed.
10. Repeat step 10 for a second elution.
11. Check the mRNA quantity by UV spectrometry. Expect 1.5 to 2.5 % of the starting amount total RNA, depending on the considered morphological stage of the parasite.
12. *Stopping point:* The mRNA solutions can be stored at -80°C. When needed, thaw tubes on ice. Avoid repeated freeze/thaw cycles.

3.5. Fragmentation of the polyA+ mRNAs

1. Reduce sample volume to 15-20 μL in a vacuum concentrator type SpeedVac® without heating. Do not let the sample dry.
2. Add 4 volumes of 5X RNA storage solution and incubate for 40 min at 98°C (*see* **Note 9**).

[8]Any loss in beads will result in a loss of material. For maximum yield, about 50 μL of sample should remain in the tube after removing the supernatant.
[9]The efficiency of this step is directly linked to the amount of starting material. If desired, the incubation time can be adjusted accordingly but should not exceed 60 min.

3. Reduce sample volume to 10 μL in a vacuum concentrator without heating. Do not let the sample dry.

4. *Stopping point:* The mRNA solutions can be stored at -80°C. When needed, thaw tubes on ice. Avoid repeated freeze/thaw cycles.

3.6. First Strand cDNA Synthesis

First strand cDNA is synthesized using the SuperScript® VILO™ cDNA synthesis kit (Invitrogen™). All reagents and buffers mentioned in this section refer to elements of the kit. Frozen items should be kept on ice after thawing.

1. In a thin-wall nuclease-free 0.2 mL PCR-grade tube, mix 3 μg of random hexamers and 1 μg of Anchored oligod$T_{(20)}$ to the fragmented mRNA in 14 μL final volume (*see* **Note 10**).

2. Incubate the tube in a pre-heated thermal cycler at 70°C for 10 min and quickly chill on ice for 5 min. Do not reduce this time.

3. On ice, add the following reagents to the tube from step 2: 4 μL of 5X VILO™ Reaction Mix, 2 μL of 10X SuperScript® Enzyme Mix (*see* **Note 11**). If you prepare multiple samples at the same time, make a master mix containing the 5X VILO™ Reaction Mix and the 10X SuperScript® Enzyme Mix and add 6 μL of it to each sample (*see* **Note 12**).

4. Gently mix the sample by flicking the bottom of the tube with fingertips. Spin, place on ice.

5. Incubate the sample in a thermal cycler using the following program: 25°C for 10 min, 42°C for 90 min, 85°C for 5 min, and hold at 4°C.

6. Remove promptly from the thermal cycler and place the tube on ice.

7. Purify 1st strand cDNA using the DNA Clean & Concentrator™ (Zymo Research):

    a. Add 100 μL of DNA Binding Buffer to the reaction mixture and mix well by pipetting up and down.

    b. Transfer to a Zymo-Spin™ Column/collection tube assembly and centrifuge 30 s at maximum speed (14000-16000 g) in a tabletop microcentrifuge. Discard the flow-through.

    c. Add 200 μL of Wash Buffer (freshly prepared with absolute ethanol, *see* **Note 13**). Centrifuge 30 s at maximum speed.

---

[10]A combination of random primers and oligos dT should always be used in experiments dealing with *Plasmodium falciparum*'s AT-rich genome to maximize the reverse transcription of all possible transcripts regardless of their GC content.

[11]The 5X VILO™ Reaction mix already contains random primers, $MgCl_2$, and dNTPs. The 10X SuperScript® Enzyme Mix includes the SuperScript® III Reverse Transcriptase (reduced RNase H activity and high thermal stability for extended synthesis), the RNAseOUT™ Recombinant Ribonuclease Inhibitor, and a helper protein proprietary to Invitrogen™.

[12]When dealing with multiple samples at the same time, the delay between the preparation of the 1st sample and the preparation of the last sample should be kept to a minimum to ensure uniformity. Do not prepare more than 8 samples at a time.

[13]As a general rule when using nucleic acid clean up and purification reagents, buffers containing ethanol should always be as fresh as possible. Aging solutions can cause dramatic losses in material.

d. Discard the flow-through and repeat c. for a second wash.

e. Transfer the Zymo-Spin™ Column to a fresh nuclease-free microcentrifuge tube.

f. Pipet 20 µL of nuclease-free water to the column matrix and let stand 1 min. Centrifuge 30 s at maximum speed to elute the nucleic acid.

g. Repeat step f.

8. Adjust sample volume to 47 µL with non-DEPC nuclease-free water (*see* **Note 14**).

3.7. Second Strand cDNA Synthesis

All reagents and buffers mentioned in this section should be made freshly. Frozen items should be kept on ice after thawing.

1. Prepare a dNTP mix containing dATP, dCTP, dGTP, and dUTP (instead of dTTP) each at 10 mM final concentration (*see* **Note 15**).

2. Chill all reagents on ice.

3. Set up the following reaction on ice and in the provided order:

First strand cDNA	47 µL
5X First Strand Buffer	2 µL
100 mM DTT	1 µL
5X Second Strand Buffer	15 µL
10 mM dNTP (w/dUTP) mix	4 µL
E. coli DNA Polymerase I 10 U/µL	4 µL
E. coli DNA Ligase 10 U/µL	1 µL
E. coli RNase H 2 U/µL	1 µL

4. Mix gently by pipetting and incubate at 16°C for 2 hours

5. Chill the reaction on ice for at least 5 min.

6. Purify ds cDNA using the DNA Clean & Concentrator™ (Zymo Research):

a. Add 375 µL of DNA Binding Buffer to the reaction mixture and mix well by pipetting up and down.

b. Transfer to a Zymo-Spin™ Column/collection tube assembly and centrifuge 30 s at maximum speed (14000-16000 g) in a tabletop microcentrifuge. Discard the flow-through.

[14]At that point, the samples can theoretically be frozen at -20°C until further processing. Empirical observations seem to indicate, however, that the performances are significantly increased when 2nd strand cDNA is synthesized immediately after 1st strand. Therefore, we do not recommend the freezing of 1st strand cDNA.

[15]The substitution of the dTTP by dUTP in the dNTP mix is critical in this protocol since it will allow using the USER™ (Uracil-Specific Excision Reagent) enzyme prior library amplification and achieving strand specificity. The USER™ enzyme will leave a nucleotide gap at the location of a uracil in the second strand of the cDNA.

c. Add 200 μL of Wash Buffer (freshly prepared with absolute ethanol, *see* **Note 13**). Centrifuge 30 s at maximum speed.

d. Discard the flow-through and repeat c. for a second wash.

e. Transfer the Zymo-Spin™ Column to a fresh nuclease-free microcentrifuge tube.

f. Pipet 6 μL of nuclease-free water to the column matrix and let stand for 1 min. Centrifuge 30 s at maximum speed to elute the nucleic acid.

g. Repeat step f.

7. Check the ds cDNA quantity by UV spectrometry and quality by visualization on a 1.2 % agarose gel electrophoresis. A smear should be easily detected (*see* **Note 16**).

8. *Stopping point:* The sample is now ds cDNA and is relatively stable. It can be stored at -20°C. When needed, thaw tubes on ice. Avoid repeated freeze/thaw cycles.

3.8. ds cDNA fragmentation

1. Mix 700 ng of ds cDNA with 11.5 μL of 3X dsDNA Shearase™ Reaction buffer and 3.5 μL of dsDNA Shearase™ (Zymo Research). Reach a final volume of 35 μL final with nuclease-free water.

2. Incubate at 37°C for 40 min (*see* **Note 17**).

3. Purify ds cDNA and inactivate the dsDNA Shearase™ by adding 175 μL of the DNA Clean & Concentrator™ DNA binding buffer (Zymo Research).

4. Mix well by pipetting up and down and transfer to a Zymo-Spin™ column/ collection tube assembly and centrifuge 30 s at maximum speed (14000-16000 g) in a tabletop microcentrifuge. Discard the flow-through.

5. Add 200 μL of Wash Buffer (freshly prepared with absolute ethanol, *see* **Note 13**). Centrifuge 30 s at maximum speed.

6. Discard the flow-through and repeat step 5 for a second wash.

7. Transfer the Zymo-Spin™ column to a fresh nuclease-free microcentrifuge tube.

8. Pipet 10 μL of nuclease-free water to the column matrix and let stand 1 min. Centrifuge 30 s at maximum speed to elute the nucleic acid.

9. Repeat step 8.

10. Check the size range and the concentration of the sample using microfluidic-based separation devices suitable for small amounts of starting materials, such as an

[16]Obtaining high quality ds cDNA is an absolute prerequisite for a successful preparation of a sequencing library. We recommend not proceeding if the ds cDNA is not of satisfactory quality (the presence of a regular smear on the gel and an OD ≥ 1.8 is an example of satisfactory quality).

[17]These reaction conditions have been optimized to obtain fragments ranging 150 bp to 300 bp in size. Increase incubation time for shorter fragments, decrease it for longer ones.

Agilent 2100 Bioanalyzer (Agilent Technologies) or a LabChip® GX (Caliper Life Sciences) (*see* **Note 18**). Repeat the fragmentation procedure if necessary.

11. *Stopping point:* The sample can be stored at -20°C. When needed, thaw tubes on ice. Avoid repeated freeze/thaw cycles.

3.9. Library preparation

The protocol described here uses the NuGEN® Encore™ NGS Library System I, compatible with the Illumina® Genome Analyzer and Hi-Seq sequencing platforms, and all mentioned reagents refer to components of this kit (*see* **Note 19**). However, since the starting material is double stranded DNA, it can be easily adapted to any gDNA library preparation kit (including multiplexing) or set of reagents.

The Agencourt® magnetic beads used for sample clean up must be incubated at room temperature for at least 15 min before use. Cold beads reduce yields. Before each use, beads must be fully resuspended by inverting and tapping the tube. Thaw all necessary reagents, mix by vortexing, spin, and keep them on ice until use. Keep the nuclease-free water at room temperature.

3.9.1. End repair

1. Dilute 200 ng of fragmented ds cDNA to a volume of 7 μL with nuclease-free water in a 0.2 mL thin-wall nuclease-free PCR tube. Place on ice.
2. On ice, add 2.5 μL of End Repair Buffer Mix and 0.5 μL of End Repair Enzyme Mix to the sample and mix by pipetting up and down. If more than one sample is treated, prepare a master mix of sufficient amounts of End Repair Buffer Mix and End Repair Enzyme Mix before adding 3 μL to each sample (*see* **Note 12**).
3. Place the tube in a pre-warmed thermal cycler (lid heated at 100°C) with the following program: 30 min at 25°C; 10 min at 70°C; hold at 4°C.
4. Remove the sample promptly from the thermal cycler, give a quick spin, and place on ice.
5. Resuspend Agencourt® RNAClean XP magnetic beads by inverting and tapping the tube on the bench top. Do not spin the tube.
6. Add 12 μL of the bead slurry to the sample and mix thoroughly by pipetting up and down. Incubate at room temperature for 10 min.

---

[18]Small amounts of nucleic acids cannot be detected by classical agarose gel electrophoresis. In order to avoid wasting large amounts of samples we recommend using microfluidic-based devices that can quantify and display the size distribution of a few microliters of a sample concentrated in the picogram per microliter range. The Bioanalyzer DNA High Sensitivity Chip (Agilent Technologies) can resolve 3 μL of purified DNA at 5 μg/μL in TE for sizes ranging 50-7000 bp. The LabChip® GX can resolve bands as low as 5 bp and features a sensitivity of 0.1 ng/μL.

[19]The NuGEN® Encore™ NGS Library System I uses magnetic beads (RNAClean® XP Purification Beads supplied in the kit) for the successive purification of the samples through the library preparation steps rather than silicate-based spin columns. Magnetic beads allows for minimized sample loss and reduction of input material for library preparation. A magnetic separation device, such as the Agencourt® SPRIStand, is thus necessary to perform the purification steps. When using the Agencourt® SPRIStand, 96-well plates or tube strips are preferred rather than single tubes for greater stability in the stand and better separation.

7. Transfer tubes to the magnetic separation device and let them stand for 5 min (*see* **Note 20**).
8. While still on the magnet, carefully pipet off 15 μL of liquid without disturbing the beads (*see* **Note 21**). Dispersion and loss of significant amounts of beads will reduce yields.
9. While still on the magnet, gently add 200 μL of freshly made 70 % ethanol and let stand for 30 s (*see* **Note 22**).
10. While still on the magnet, remove 200 μL of the ethanol wash (*see* **Note 23**).
11. Repeat step 9.
12. While still on the magnet, remove all of the ethanol wash. Carefully inspect the tube for the absence of ethanol drops.
13. While still on the magnet, air-dry the beads for 5-10 min. Carefully inspect the tube to ensure the ethanol has entirely evaporated.
14. Remove from the magnet and add 12 μL of nuclease-free water to the dried beads. Resuspend carefully by pipetting up and down.
15. Transfer the tubes to the magnet and let them stand for 1 min.
16. While on the magnet, carefully remove 11 μL of the eluate without disturbing the beads and transfer to a fresh tube.
17. Repeat step 15 to minimize the carry over of beads into the next stage of the library preparation.
18. While on the magnet, carefully remove 10 μL of the eluate without disturbing the beads and transfer to a fresh nuclease-free thin-wall 0.2 mL PCR tube. Place on ice.
19. Proceed immediately to “3.9.2. Ligation” (*see* **Note 24**).

3.9.2. Ligation

1. On ice, add 1 μL of Adaptor Mix to the sample (*see* **Note 25**). Mix by pipetting thoroughly with the pipette set to 5 μL.

---

[20]Reduction in the incubation time of the beads on the magnetic stands will result in reduced recovery of the samples. Similarly, the various incubation times have been optimized to obtain reproducible results in terms of nucleic acid yield and size range. They must be strictly observed.

[21]While on the magnet, the beads will stay on the walls of the tube and form a ring. Use a small volume pipette tip to reach the bottom of the tube without touching the sides and gently aspirate the desired volume.

[22]If multiple samples are treated simultaneously, monitor the time spent in reaching the last tube and deduct it from the 30 s.

[23]Always use the smallest volume pipette tip that allows the removal of the desired volume within 2 to 3 withdrawals. Do not try to get everything in one-step and pipet slowly to prevent any bead loss.

[24]NuGEN® developed proprietary adapter and primer sequences directly compatible with the Illumina® Genome Analyzer and Hi-Seq systems. Their use differ from the more common Illumina® ones mostly in the fact that the step for 3’-end A-tailing of the fragments that is usually carried on prior adapter ligation is absent in the NuGEN® protocol. This specificity significantly reduces the hands-on time of the protocol. In addition, NuGEN® adapters generate libraries free from adaptor dimers, unlike Illumina®'s adapters.

[25]The adapters are partly complementary and provided partially annealed to each other. This condition is necessary for a successful ligation to the sample of interest. Make sure to always keep the tube of Adapter Mix on ice so that the adapter duplex does not denature.

2. On ice, add 12.5 μL of Ligation Buffer Mix and 1.5 μL of Ligation Enzyme Mix to the sample (the Ligation Buffer Mix is very viscous and should be pipetted slowly). If more than one sample is treated, prepare a master mix of sufficient amounts of Ligation Buffer Mix and Ligation Enzyme Mix before adding 14 μL to each sample (*see* **Note 12**).
3. Carefully mix by pipetting slowly up and down without forming bubbles with the pipette set at 20 μL. Spin down the tube for 2 s.
4. Place the tube in a pre-warmed thermal cycler (lid not heated) with the following program: 10 min at 25°C; hold at 4°C. **IMPORTANT**: Use this incubation time to prepare the Amplification Master Mix to be used in the library amplification reaction (*see* section 3.9.3. step 1). The adapter-ligated sample must not remain on ice more than 10 min from the end of the ligation reaction to the beginning of the amplification reaction.
5. Remove the sample promptly from the thermal cycler, give a quick spin, and place on ice.
6. Proceed immediately to “3.9.3. Library Amplification”.

3.9.3. Library Amplification

1. Prepare an Amplification Master Mix by sequentially mixing the following reagents: 64 μL of Amplification Buffer Mix, 3 μL of Amplification Primer Mix, 4 μL of DMSO (this mix should have been prepared during the incubation indicated at section 3.9.2. step 4). Place tube on ice. If more than one sample is treated, adapt volumes to prepare a sufficient quantity of master mix.
2. On ice, add 3 μL of Amplification enzyme mix and 1 μL of USER™ enzyme to the Amplification Master Mix immediately before adding to the adapter-ligated sample (the USER™ enzyme will degrade the second strand of the ds cDNA prior amplification to achieve strand specificity, *see* **Note 26**). If more than one sample is treated, adapt volumes to prepare a sufficient quantity of master mix.
3. Mix well by pipetting slowly, avoiding bubbles, spin, and place on ice.
4. Add 73 μL of Amplification Master Mix to a clean 0.2 mL thin-wall nuclease-free PCR tube.
5. Add 7 μL of adapter-ligated sample to the tube prepared at step 4. Mix well by pipetting slowly up and down at the 73 μL pipette setting, avoiding bubbles, spin, and place on ice. The remaining adapter-ligated sample can be discarded.
6. Place the tube in a pre-warmed thermal cycler (lid heated at 100°C) with the following program: 5 min at 95°C; 2 min at 72°C; 5 cycles of (30 s at 94°C – 30 s

[26]The USER™ (Uracil-Specific Excision Reagent) enzyme is added to the amplification mix. During a short denaturing step (5 min at 95°C) prior to the actual amplification cycles, the USER™ enzyme nicks the second strand of the ds cDNA at uracil locations. Only the first strand is amplified and strand specificity is achieved.

at 55°C – 1 min at 72°C); 10 cycles of (30 s at 94°C – 30 s at 63°C – 1 min at 72°C); 5 min at 72°C; hold at 10°C.

7. Remove the sample promptly from the thermal cycler, give a quick spin, and place on ice.

8. Resuspend Agencourt® RNAClean XP magnetic beads by inverting and tapping the tube on the bench top. Do not spin the tube.

9. Add 80 μL of the bead slurry to the amplified library and mix thoroughly by pipetting up and down (*see* **Note 27**). Incubate at room temperature for 10 min.

10. Transfer tubes to the magnetic separation device and let them stand for 5 min (*see* **Note 20**).

11. While still on the magnet, carefully pipet off 140 μL of liquid without disturbing the beads (*see* **Note 21**). Dispersion and loss of significant amounts of beads will reduce yields.

12. While still on the magnet, gently add 200 μL of freshly made 70 % ethanol and let stand for 30 s (*see* **Note 22**).

13. While still on the magnet, remove 200 μL of the ethanol wash (*see* **Note 23**).

14. Repeat step 12 to 13 two more times for a total of three washes.

15. While still on the magnet, remove all of the ethanol wash. Carefully inspect the tube for the absence of ethanol drops.

16. While still on the magnet, air-dry the beads for at 10-15 min. Carefully inspect the tube to ensure the ethanol has entirely evaporated.

17. Remove from the magnet and add 33 μL of 1X TE to the dried beads. Resuspend carefully by pipetting up and down.

18. Transfer the tubes to the magnet and let stand for 2 min.

19. While on the magnet, carefully remove 30 μL of the eluate without disturbing the beads and transfer to a fresh tube. Place on ice.

20. *Stopping point:* The amplified libraries can be stored at -20°C. When needed, thaw tubes on ice. Avoid repeated freeze/thaw cycles.

3.9.4. Qualitative and Quantitative Evaluation of the Library

1. Analyze 3 μL of the library on a 1.6 % agarose gel electrophoresis (*see* **Note 28**) and check the size and the purity of the library. Quantify by UV spectrophotometry.

[27] If multiple samples are processed at the same time, it may be useful to use a multi-channel pipette to ensure consistent incubation times.

[28] A high percentage of agarose is necessary to resolve small libraries. Increasing the amount of agarose, however, significantly increases the detection threshold using intercalant agents such as ethidium bromide. Here, preparing a gel at 1.5-1.8 % agarose is a good compromise between resolution and sensitivity. In addition, the use of low range agarose, such as the Certified Low Range Ultra Agarose (Bio-Rad), greatly improves the resolution of small bands without having to increase the agarose content.

Acknowledgments

The authors thank Courtney Brady (NuGEN®), and Barbara Walter, John Weger, Rebecca Sun, and Glenn Hicks (Institute for Integrative Genome Biology, University of California Riverside) for their assistance in the library preparation and sequencing processes.

References

1. Le Roch, Karine G., et al. Discovery of gene function by expression profiling of the malaria parasite life cycle. Science (New York, N.Y.). Sep; 2003 30112(5639):1503–1508.
2. Bozdech Z, et al. The transcriptome of the intraerythrocytic developmental cycle of Plasmodium falciparum. PLoS Biol. Oct.2003 1(1):E5. [PubMed: 12929205]
3. Otto, Thomas D., et al. New insights into the blood-stage transcriptome of Plasmodium falciparum using RNA-Seq. Molecular Microbiology. Apr; 2010 76(1):12–24. [PubMed: 20141604]
4. Sorber, Katherine; Dimon, Michelle T.; DeRisi, Joseph L. RNA-Seq analysis of splicing in Plasmodium falciparum uncovers new splice junctions, alternative splicing and splicing of antisense transcripts. Nucleic Acids Research. May; 2011 39(9):3820–3835. [PubMed: 21245033]