**Protocol for VSG RNA-seq (Nextera XT kit)**

*Last updated: Monica Mugnier, December 16, 2016*

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2.1 DNase treatment protocol

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**1. RNA extraction**

Isolating the total RNA from *Trypanosoma brucei* cells

**1.1** RNA extraction protocol

*Adhere to strict RNase-free technique*

1. Harvest cells, spin down and remove supernatant
2. Resuspend pellet in 1mL RNA STAT-60, pipette up and down to lyse cells
3. Incubate at room temperature for 5 minutes
4. Add 200µL chloroform and shake by hand vigorously for 15s
5. Incubate at room temperature for 3 minutes
6. Centrifuge for 15 mins at 12,000g/4’C
7. Transfer aqueous (upper clear) phase to a fresh tube, the volume should be roughly 500µL
8. Add 500µL isopropanol and incubate at <4’C for at least 30 mins
9. Centrifuge for 15 mins at 12,000g/4’C
10. Remove supernatant, wash pellet with 500µL 75% ethanol

* Pellet may look invisible, but it is there.

1. Centrifuge for 5 mins at 12,000g/4’C
2. Remove supernatant (may be necessary to spin again for 1 min and remove remaining supernatant with needle tip)

* Pellet may look invisible, but it is there.

1. Allow pellet to air-dry for 5 mins
2. Resuspend in 30µL water
3. Store sample at -80’C

Following completion of the protocol determine concentration of RNA in sample (blank with water) with nanodrop.

**2. DNase treatment**

Removing/minimizing DNA contamination in RNA sample

**2.1** DNase treatment protocol

*Adhere to strict RNase-free technique*

1. For every *x* µg RNA set up following reaction

*(if* ***x*** *< 3.5, add 3.5 of both buffer and enzyme for a total of 35µL)*:

28.5µL RNA (from section **1.1**)

***x*** µL RQ1 DNase

***x***µL 10X reaction buffer

Adjust volume to 10 × ***x***µL with water

*Do not vortex reaction*

1. Incubate reaction at 37’C for 30 mins

\*\* If preparing to store:

* After previous incubation, add:

***x*** µL RQ1 Stop Solution

* Incubate reaction at 65’C for 10 mins

This can be stored at -80’C

**2.2 Reaction clean-up (Ampure RNAclean XP)**

*Adhere to strict RNase-free technique*

1. Resuspend beads, bring to room temp
2. Add 1.8 × (*µl of RNA)* of beads to sample.
3. Pipette 10x to mix, and incubate for 5-20 min at room temp without shaking.
4. Place tube on magnet for 2 min.
5. Remove supernatant.

* Hold tube securely when opening anytime on magnet so contents doesn’t splash.

1. Wash 2x by dispensing 200 µl 70% ethanol (fresh) for 30s, and removing. Do not remove the tube from the magnetic stand.
2. Allow pellet to dry briefly (no more than 5 minutes), but do not overdry
3. Remove tube from magnet and add 32l H2O and pipette up and down 10x to mix.
4. Place on magnet for 1 min
5. Transfer eluate (30 µl) to a fresh (1.5ml)tube.

This can be stored at -80’C

**3. First strand synthesis**

Using the reverse transcriptase enzyme Superscript III to produce first strand cDNA

**3.1** First strand synthesis protocol

*Adhere to strict RNase-free technique*

1. If input is <20µg RNA, set up the following reaction, in PCR tubes:

8.0µL RNA (from section **2.2**)

1µL 2mM All-VSG 3’ UTR primer

1µL dNTP mix

1. If input for section **2.** is >20µg RNA, scale up reaction proportionally.
2. Incubate at 65’C for 5 mins, and cool on ice for at least 1 minute
3. For every 10µL of previous reaction, make the following synthesis mix separately:

2µL RT buffer

4µL 25mM MgCl2

2µL 0.1M DTT

1µL RNaseOUT (40U/µL)

1µL Superscript III RT

1. Add synthesis mix to starting reaction and briefly centrifuge
2. Incubate for 10 mins at 25’C, followed by 50 mins at 50’C
3. Terminate reaction by incubating sample for 5 mins at 85’C
4. Add 1 µL RNaseH and 1 µL RNase A (qiagen kit) to each tube and incubate for 30 min at 37’C

• Sample can be stored at -20’

**3.**2 Reaction clean-up (AMPure RNAclean XP)

1. Resuspend beads, bring to room temp
2. Add 1.8x beads (40 µl) to sample.
3. Pipette 10x to mix, and incubate for 5-20 min at room temp without shaking.
4. Place tube on magnet for 2 min.
5. Remove supernatant.
6. Wash 2x by dispensing 200 µl 70% ethanol (fresh) for 30s, and removing. Do not remove the tube from the magnetic stand.
7. Allow pellet to dry briefly (no more than 5 min), but do not overdry
8. Remove tube from magnet and add 33l H2O and pipette up and down 10x to mix.
9. Place on magnet for 1 min
10. Transfer eluate (32 µl) to a fresh tube. → put into PCR tubes if immediately continuing.

**4. PCR amplification of VSGs**

Synthesizing ds-cDNA from the cDNA produced in first strand synthesis (Phusion polymerase)

**4.1** Second strand synthesis protocol

1. Set up the following reaction on ice:

32 µL cDNA from section **3.2**

10 µL HF buffer

1 µL 10 mM dNTPs

2.5 µL 10 µM 14mer-SP6 oligo

2.5 µL 10 µM SL oligo

* 1. µL DMSO

0.5 µL Phusion DNA Polymerase

0 µL H2O

1. Put in thermocycler with following program

98ºC 30 sec

**22** cycles of:

98ºC 10s

55ºC 10s

72ºC 45s

72ºC 5 min

\*if you are concerned about yield, run a small aliquot(5ul of sample) on a gel to check for a band

**4.2** Reaction clean-up (AMPure XP)

1. Resuspend beads, bring to room temp
2. Add 0.7x beads (35 µl) to sample.
3. Pipette 10x to mix, and incubate for 5 min at room temp without shaking.
4. Place tube on magnet for 2 min.
5. Remove supernatant.
6. Wash 2x by dispensing 200 µl 70% ethanol (fresh) for 30s, and removing. Do not remove the tube from the magnetic stand.
7. Dry pellet briefly (no more than 5 min) but do not overdry
8. Remove tube from magnet and add elution buffer (40 µl is ideal – use 30µl for this) and pipette up and down 10x to mix.
9. Place on magnet for 1 min
10. Remove eluate (remove 28µl)
11. Quantify DNA using Qubit HS dsDNA assay

**5. NextEra Tagmentation**

**5.1** Tagmentation of Input DNA

1. Thaw **ATM, TD,** and DNA on ice. Ensure **NT** is at room temp and there is no precipitate.
2. In 0.2ml tube, add 10 µl of **TD Buffer**
3. Add 5 µl of input DNA at 0.2 ng/µl (1ng total)
4. Add 5 µl of **ATM**
5. Pipette mixture up and down 5x to mix
6. Centrifuge sample at 280g at 20ºC for 1 minute
7. Place sample in thermocycler and run program:
   1. 5 min 55ºC
   2. hold 10ºC
8. Once sample reaches 10ºC proceed to neutralization (6.2)

**5.2** Neutralization of Tagmentation reaction

1. Add 5 µl of **NT Buffer** to sample
2. Pipette up and down 5x to mix
3. Centrifuge at 280g at 20ºC for 1 minute
4. Place sample at room temperature for 5 min

**6. PCR amplification**

**6.1**  PCR amplification of tagmented sample

1. Add 15µl of **NPM** to tagmented sample
2. Add 5 µl of **each index primer** to sample
   1. RECORD INDICES USED!
   2. MAKE SURE PAIRING IS CORRECT
3. Pipette up and down 5x
4. Centrifuge at 280g at 20ºC for 1 minute
5. Perform PCR using the following program:

72ºC 3 min

95ºC for 30s

12 cycles of:

95ºC 10s

55ºC 30s

72ºC 30s

72ºC 5 min

Hold at 10ºC

**6.2** PCR clean-up (AMPure XP)

1. Centrifuge sample after PCR, 180g, 20ºC, 1 min
2. Transfer sample to 1.5ml eppendorf tube
3. Add 50µl (1.0x) beads (This has been changed by illumina! now recommending 0.6x beads, 30µl)
4. Mix by pipetting up and down 10 times
5. Incubate at room temperature for 5 min
6. Place tube on magnet for 2 min. Remove supernatant.
7. Wash beads 2x with 200µl (fresh!) 80% EtOH. Do not resuspend beads. Leave sample for 30s, then remove supernatant.
8. Allow beads to air dry briefly, but do not overdry
9. Remove tube from magnet and add 27µl of RSB to tube.
10. Mix by pipetting up and down, and then incubate at room temperature for 2 min.
11. Place tube on magnet for 2 min.
12. Transfer 25µl of PCR amplicon to new tube.