IF IT WORKS, IT IS NOT STUPID.

BUSTA LAB MOTTO 1

JUST BECAUSE SOMETHING HAS ALWAYS BEEN DONE ONE WAY DOES NOT MEAN IT CAN'T BE IMPROVED.

BUSTA LAB MOTTO 2

THE BUSTA LAB

THE BUSTA LAB HANDBOOK

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Contents

List of Figures

List of Tables

Dedicated to those who appreciate LaTeX and the work of Edward R. Tufte and Donald E. Knuth.

Nucleic Acids

Tissue Harvesting and Homogenization

Tissue harvesting and homogenization
[ ] Isolate tissue with RNAse-free tweezers and scissors, put in 50 ml falcon tube, flash freeze.
[ ] Transfer small aliquots of tissue from 50 ml falcon tube to mortar, grind to a *FINE* powder, liquid N2 as needed.
[ ] Use pre-labelled pre-frozen 2 ml tubes to scoop and store powder, always keeping tissue frozen. Store at -80°C.

(m)RNA Extraction

Precipitation of total RNA

Dissolve nucleotides and remove cellµlar debris

[ ] Clean bench: Add CTAB (20 ml) to two 50 ml tubes. Incubate in water bath (65°C, 5 min).

[ ] Fume hood: add β-mercaptoEtOH (400 μl), and *frozen* tissue (1-2 ml powder). Vortex 30s.

[ ] Incubate in water bath (65°C, 5 min).

[ ] Clean bench: balance tubes in pairs using nuclease-free water. Centrifuge (20k g, 5 min, 4C).[1]

[ ] Fume hood: decant supernatants into new falcon tubes. Discard old falcon tubes in biohazard waste.

Separate RNA from DNA and Protein

[ ][ ] 2x: Fume hood: add phenol:$CHCl_3$:IAA (0.2 vol (1)), cap & vortex (15 s), balance tubes with nuc-free water, centrifuge (10k g, 10 min, 4°C), upper phases to new tubes, old tubes to biohazard waste.[2]

[ ] Fume hood: decant upper phase to new tubes (50ml falcons). Old falcons to biohazard waste.

Precipitate RNA

[ ] Clean bench, add LiCl (0.30 vol/sample) to the falcon tubes, cap, vortex (15 s) Balance tubes with RNase-free water.[3]

[ ] Incubate at -20°C for 16 hours to precipitate RNA. ..... **$**

RNA collection (2 hours)

Collect RNA (1.5 hours)

[ ] Chill the centrifuge and rotor to 4°C. Fume hood: balance tubes with RNase-free water. Spin (20k g, 1 hr, 4°C).

[ ] Wipe off condensation with kimwipe, mark pellet with marker, aspirate supernatant, pipette off excess.

[ ][ ] 2x: Wash pellet in cold 80% EtOH (1ml), Spin (20k g, 1 min, 4°C), pipette off EtOH, dry in clean bench[4].

[ ] Resuspend each pellet in RNase-free water (100 μl), these are your RNA isolates.

Quantify and QC the RNA isolates (20 minutes)

[ ] Make *WorkingSolution*[5] (3 samp + 2 stds): In a 1.5 mL Lo-bind: 5μl Reag., 995μl buffer.

[ ] In Qubit tubes, RNA: 190μl *WorkingSolution*, 10μl RNA. Stds: 190 μl *WorkingSolution*, 10 μl std.

[ ] Vortex, then wait 2 minutes (important!), then measure. Record concentrations (ng/μl) in table at right.

[ ] Make IQ WS: (for 3 stds + 1 samp.) In a 1.5 ml Lo-Bind tube (1194 μl buffer + 6 μl reagent), stds (10μl std + 190 μl WS), and sample (10 μl RNA + 180 μl IQ WS).

[ ] Vortex 15s, wait 2 min (Important!). Record IQ for each RNA isolate in the table at right. Store -80°C. ..... **$**

At this point you need > 50μl of 100ng/μl to proceed.

Isolate mRNA (3 hours, requires 50μl of 100ng/μl total RNA = 5ug RNA)

Wash beads

[ ] Put beads at RT 30 min, vortex periodically. Preheat PCR machine to 65°C. RNase-Zap bench area & pipettes.

[ ] Add NEBNext Magnetic Beads (20μl) to a 0.2 ml PCR tube.

[ ][ ] 2x: Add RNA Binding Buffer (100μl), mix 6x [2s up, 2s down], pellet on magnet 3 min, discard supernat., remove from magnet.

Bind mRNA to beads

[ ] Dilute 5ug total RNA to 50μl in 1.5ml LoBind, add RNA binding buffer (50μl), mix 6x [2s up, 2s down].

[ ] Using the PCR machine, incubate the tubes (65°C, 5 min), then bring to 4°C.

[ ][ ] 2x: Once 4°C, remove tubes, set PCR machine to 80°C, mix by pipetting 6x [2s up, 2s dn], incubate (5 min, RT).

[ ] Place tubes on magnet for 5 min, remove and discard supernatant, remove tubes from magnet.

[ ] Add RNA Wash Buffer (200μl), mix 6x [2s up, 2s down], pellet on magnet 3 min, discard supernatant.

Release and rebind mRNA

[ ] Add 50μl TRIS, mix 6x [2s up, 2s dn], incubate in preheated PCR machine (80°C, 2 min), then cool to 25°C.

[ ] Once 25°C, remove tubes, preheat PCR machine to 80°C, add 50μl RNA binding buffer, mix 6x [2s up, 2s down].

[ ] Incubate at room temp 5 minutes, resuspend by mixing 6x [2s up, 2s down], incubate at room temp 5 minutes.

[ ] Plate tubes on magnet for 3 minutes, remove and discard supernatant, remove tubes from magnet.

Wash beads once, removing ALL supernatant

[ ] Add Wash buffer (200μl) and mix 6x [2s up, 2s down], place tubes on magnet for 5 minutes.

[ ] Remove, discard supernatant, spin tubes, tubes to magnet (2 mins), discard ALL supernatant, tubes off magnet.

Elute mRNA

[ ] Add 17μl TRIS, mix 6x [2s up, 2s down], incubate in preheated PCR machine (80°C, 2 min, then cool to 25°C.)

[ ] Once 25°C, immediately put tubes on magnet. Transfer supernatant only (no beads) to clean tubes on ice.

Measure mRNA concentration with the Qubit (do not touch sides of qubit tubes during use!)

[ ] Make WS: 3.5μl Reagent, 696.5μl buffer. In Qubit tubes: S1: 190μl WS, 10μl Std1; S2: 190μl WS, 10μl Std2; RNA: 196μl WS, 4μl RNA

[ ] Vortex, then wait 2 minutes (important!), then measure. Record concentrations (ng/μl). $

[1] All debris should be pelleted, if not, spin longer.

[2] Acidic phenol:$CHCl_3$:IAA separates RNA from protein *and* DNA, enabling precipitation with isopropanol as opposed to reverse transcription-inhibiting LiCl. This (and later mRNA isolation) also obviates DNase treatment.

[3] LiCl precips RNA > 300 nt in length.

[4] EtOH precips RNA. Taylor showed EtOH washes help RT, at least, when LiCl is used to precipitate.

[5] Do not touch tube sides while using qubit. Use RNA "HS" High Sensitivity Kit and RNA "IQ" Kit.

Sample	ng/μl	μl	IQ

Sample	ng/μl	μl	IQ

mRNA Sequencing

cDNA library preparation (7 hours)

Reverse Transcription

[ ] *Mix 1*: 1 ng mRNA (x μl), 1 μl VNP, 1 μl dNTPs, 9-x μl RNase-free water, FMSD[6], 65°C 5 min, snap freeze.[7]
[ ] *Mix 2*: 4 μl RT buffer, 1 μl RNaseOUT, 1 μl RNase-free water, 2 μl SSP, FMSD.
[ ] Make *RT Mix 1*: Add Mix 2 to Mix 1, FMSD, Incubate (42°C, 3 min).
[ ] Make *RT Mix 2*: Add 1 μl RT enzyme[8] (200 U/μl) to RT Mix 1. FMSD. Incubate (42°C, 90 min).
[ ] Inactivate *RT Mix 2* by incubating (85°C, 5 min; hold at 4°C).
** Is there some way to run QC on length distribution here???
I abandoned cDNA kit because I was getting an abundance of short reads 150bp to 300bp that I didnt even see with direct RNA seq which doesn't have a strand switch oligo to select full length transcripts'.
I ended up switching to the direct RNA kit, which confirmed it wasn't the input RNA's fault as the read length counts distribution looked great compared to direct cDNA. And the distribution was correlated well with what I saw on the bioanalyzer. Just in general Nanopore need to do more on the RNA seq side of things.
Reducing PCR cycles (if possible) can be useful, PCR tends to favour shorter amplicons as they amplify more efficiently. So doing as few cycles as possible can help give you a higher proportion of longer reads.

[6] FMSD = flick mix and spin down
[7] on block
[8] Superscript Reverse Transcriptase IV or Maxima H Minus Reverse Transcriptase

PCR amplification (2 hours)

[ ] Make 4 identical tubes of *Mix 3*: 18.5 μl nuc-free water, 25μl 2x LongAmp MM, 1.5μl cPRM, 5μl RT Mix 2. FMSD.
[ ] Run PCR on *Mix 3*: [95C - 1 min], [95°C, 15s | 62°C, 15s | 65°C, 150s] x 30 cycles[9], [65°C, 6 min], [4°C hold]. $
[ ] Add 1μl Exonuclease (20 U) to each tube of *Mix 3*, FMSD, 37°C - 15 min, 80°C - 15 min (degrades primers).

[9] Nanopore says 11-22 cycles, start with 14. We find 30 is usually necessary, though too many cycles can create "PCR duplicates" apparently

Cleanup and Concentrate cDNA (1 hour)

[ ] Resuspend beads by vortexing for 1 min and let them warm to *room temperature*.
[ ] Pool Mix 3s in new 1.5ml LoBind. Vortex beads (10 s), add 120 μl beads to Mix 3.[10] Rock (5 min, RT).
[ ] Prepare 500 μl fresh 70% ethanol using RNase-free water (350 μl EtOH + 150 μl water).
[ ] Pellet beads on magnet at least 5 minutes [DO NOT spin down], remove supernatant. Prepare gel for quality check.
[ ][ ][ ] 3x: Tube on magnet, add 1000 (/enough to cover pellet) μl 70% EtOH, beads sit in EtOH for 30s, discard EtOH in two batches.
[ ] Tube on magnet: with a P10, remove residual EtOH, then dry for 30 seconds.
[ ] Remove tube from rack, resuspend gently by pipetting in 12μl Elution Buffer. Incubate RT 10 minutes.
[ ] Pellet beads on magnet 5 min [DO NOT spin down] remove + retain 12μl of eluate in new 1.5 mL LoBind, spent beads to biohazard.
[ ] Analyze 1μl of amplified cDNA for size, quantity, quality using Qubit and gel: ng/μl, μl total, and bp avg size. $

[10] Bead:DNA ratio is critical!! 120μl of beads is a 0.6 ratio which should give 400-500bp+ in the eluate. 100μl of beads is a 0.5 ratio which should give 600bp+ in the eluate. Other ratios are also possible.

Sequencing

Rapid Adapter Addition

[ ] Thaw Sequencing buffer (SQB), Loading Beads (LB), Flush tether (FLT), and one tube flush buffer (PFB/FB).
[ ] Make up 100-200 fmol cDNA in Elution Buffer (EB; final vol. 12μl). At 1kb, 100 fmol = 60 ng, 200 fmol = 120 ng.
[ ] Add Rapid Adapter (RAP; 1 μl), mix by pipetting, spin down. Incubate (10+ min RT). 4.2 On MinION Flowcell Run on Nanopore flowcell (1 hr + up to 48 hours runtime)

Sequencing on MinION

[ ] Set P1000 to 200 μl and insert tip into priming port. Using pipette suction method, turn the dial to 220-230 μl, or until you can see a small amount of buffer entering the pipette tip. [ ] Prepare the flow cell priming mix: 30 μl of thawed and mixed Flush tether (FLT) directly to the thawed and mixed Flush buffer (FB). Mix by vortexing. [ ] With SpotON closed, add 800μl PriMix to the priming port without introducing air into the system. Wait 10 minutes. [ ] Add 37.5μl SQB to the cDNA, thoroughly mix Loading Beads by pipetting, add 25.5 μl beads to 12 μl cDNA to make Sample. [ ] Add 200μl PriMix to the priming port without introducing air into the system. [ ] Mix the prepared library gently by pipetting just prior to loading. [ ] Open SpotON, add 75μl Sample dropwise to the SpotON port. Ensure each drop flows into the cell before adding the next. Close SpotON, close priming port, close lid.

Sequencing on Flongle To load the Flongle flow cell, 7.5 μL of sequencing buffer (Oxford Nanopore Technologies), 5 μL of freshly mixed loading beads (Oxford Nanopore Technologies), and 2.5 μL of the prepped DNA were mixed in a new 1.5 mL Eppendorf tube.
Following priming, the freshly mixed solution of sequencing buffer, loading beads, and DNA library was added in a dropwise fashion to the sample port of the flow cell, and a new sequencing run was started. If pore occupancy was initially low (i.e. < 10 pores sequencing), a second library (again consisting of 7.5 μL sequencing buffer, 5 μL loading beads, and 2.5 μL DNA) was added to increase sequencing yield.

Materials and Reagents

Reagents

dNTPs
(10mM)

Nanopore Primers

VNP
(2uM) 5′ - 5phosACTTGCCTGTCGCTCTATCTTCTTTTTTTTTTTTTTTTTTTTVN - 3′ Where V = A, C, or G, and N = A, C, G, or T The 5′ phosphate is added as a modification during oligo synthesis for improved ligation efficiency. However, this phosphate is not essential for the Direct cDNA Sequencing protocol, since the end-repair step also adds a 5′ phosphate.
SSP
5′ - TTTCTGTTGGTGCTGATATTGCT mGmGmG - 3′
cPRM
Forward: 5′ - atcgcctaccgtgacaagaaagttgtcggtgtctttgtgACTTGCCTGTCGCTCTATCTTC - 3′ 5′ - atcgcctaccgtgacaagaaagttgtcggtgtctttgtgAGCTAGcACTTGCCTGTCGCTCTATCTTC - 3′ Reverse: 5′ - atcgcctaccgtgacaagaaagttgtcggtgtctttgtgTTTCTGTTGGTGCTGATATTGC - 3′ 5′ - atcgcctaccgtgacaagaaagttgtcggtgtctttgtgAGCTAGcTTTCTGTTGGTGCTGATATTGC - 3′
TO MAKE CUSTOM BARCODES, INSERT CUSTOM SEQUENCE BETWEEN CAPS/LOWER CASE ABOVE

Bibliography

[1] Piotr Chomczynski and Nicoletta Sacchi, "The single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction: twenty-something years on", *Nature Protocols*, 1(2), 2006, pp. 581–585.