August 2021

REVOLUGEN

Index

!IMPORTANT NOTES!

Figure 1. White blood cells from 1ml of horse blood were processed according to updated protocol A.

Figure 2. White blood cells from 1ml of horse blood were processed according to lysate fragmentation protocol D.

Figure 3. White blood cells from 1ml of horse blood were processed according to lysate fragmentation protocol D.

A. UPDATED MAMMALIAN PROTOCOL

Up to 3-5 million mammalian cells, or 1ml of whole blood

1. Add 5X blood volume of ice-cold RBC lysis solution (100mM NH_4Cl, 10mM $NaHCO_3$, 0.1mM EDTA, pH 7.3) to up to 1ml of whole blood and vortex to mix thoroughly. ***For all other mammalian cell types pellet the cells. Remove all supernatant, after washing 1x in 1x PBS (do not wash in TE as this could have a negative effect on yield) and go straight to step 9.***
2. Incubate at RT for 5-10mins; vortex 3-5 times during incubation (for samples with volumes that fill more than half of the tube, invert before immediately placing upright onto vortex).
3. Spin at 250 x G for 3min and discard supernatant by gently tipping into waste.
4. Add 2X blood volume of ice-cold RBC lysis solution; vortex gently on lowest setting for 10s.
5. Spin at 250 x G for 3 min.
6. Pipette foam off the top of the supernatant and gently tip the supernatant into the waste; allow each pellet to rest on ice so that the excess supernatant pools.
7. Using a pipette carefully remove excess supernatant; invert tube as you draw the pipette.
8. Keep pellets on ice until use (use as soon as possible, keep on ice no longer than 1hr).
9. Just before use make a stock of 300µl of LSDNA, plus 20µl of Proteinase K, plus 10µl of RNase A (Sigma-Aldrich #R6148 is recommended) per sample by vortexing for 10secs at max speed. Stock solution needs to be made fresh right before use and not kept on ice.
10. Add 330µl of the stock solution to each sample and mix by 5x aspirations with a p1000 (narrow bore tip, set at 400µl) followed by 5secs of vortexing at max speed.
11. Incubate at 56°C for 10mins.
12. Add 350µl BS, vortex for 3secs at max speed. The solution will turn cloudy.
13. Add 400µl 75% isopropanol, vortex for 3secs at max speed. The solution will turn clear; if not manually rotate.
14. Load 650µl to the column using a p1000 wide bore tip.
15. Centrifuge at 8000rpm (4,722 x G) for 1min, discard flow through.
16. Load remaining volume to the column using a p1000 wide bore tip.
17. Centrifuge at 8000rpm (4,722 x G) for 1min, discard flow through.
18. Add 500µl WS to the column.
19. Centrifuge at 8000rpm (4,722 x G) for 1min, discard flow through.
20. Add 500µl 90% ethanol to each column.
21. Centrifuge at 14000rpm (14,462 x G) for 3mins, discard flow through.
22. Centrifuge again at 14000rpm (14,462 x G) for 1min (dry spin), discard flow through and collection tube.
23. Add inner columns to nuclease free 1.5µl tubes (pre-warmed to 80°C), incubate at 80°C for 1min with caps open (this will evaporate traces of alcohol, do not exceed 1min).
24. Add 100µl EB (pre-warmed at 80°C) to inner columns, close caps, incubate for 1min at 80°C (do not exceed 1min).
25. Centrifuge columns at 4000rpm (1,180 x G) for 2mins (Fraction A).
26. Add inner columns to new nuclease-free 1.5ml tubes (pre-warmed to 80°C), add 80µl EB (pre-warmed at 80°C) to inner columns, close caps, incubate for 1min at 80°C (do not exceed 1min).
27. Centrifuge columns at 4000rpm (1,180 x G) for 2min (Fraction B).

B. UPDATED GRAM POSITIVE CELLS PROTOCOL

Up to 1 to 1.5ml O/N culture

1. Centrifuge 1 to 1.5ml of O/N culture at 11000rpm (8,928 X G) for 3mins to pellet the cells, then remove supernatant with a p200 tip and repeat spin with 1ml of 1x PBS.
2. Snap-freeze pellets at -80^{0}C for about 5-10mins to produce greater yields.
3. Add 100µl of 20mg/ml lysozyme solution (1.2% triton X-100, 20mM Tris-HCl, pH8, 2mM EDTA) to each pellet, resuspending by 5x aspirations (p200 set to 100µl, narrow bore tip) followed by 5secs of vortexing at max speed.
4. Incubate at 37^{0}C for 30mins.
5. During lysozyme incubation make a stock of 300µl of LSDNA, plus 20ml of Proteinase K, plus 20ml of RNase A (Sigma-Aldrich #R6148 is recommended) per sample by vortexing for 10secs at max speed. Stock solution needs to be made fresh right before use and not kept on ice.
6. After lysozyme incubation add 340µl of the stock solution to each sample and mix by 5x aspirations with a p1000 (set to 400µl, narrow bore), followed by 5secs of vortexing at max speed.
7. Incubate at 56^{0}C for 20mins.
8. Add 350µl BS, vortex for 3secs at max speed. The solution will turn cloudy.
9. Add 400µl 75% isopropanol, vortex for 3secs at max speed. The solution will turn clear; if not manually rotate the tubes until it does.
10. Load 650µl to the column using a p1000 wide bore tip.
11. Centrifuge at 8000rpm (4,722 X G) for 1min, discard flow through.
12. Load remaining volume to the column using a p1000 wide bore tip.
13. Centrifuge at 8000rpm (4,722 X G) for 1min, discard flow through.
14. Add 500µl WS to the column.
15. Centrifuge at 8000rpm (4,722 X G) for 1min, discard flow through.
16. Add 500µl 90% ethanol to each column.
17. Centrifuge at 14000rpm (14,462 X G) for 3mins, discard flow through.
18. Centrifuge again at 14000rpm (14,462 X G) for 1min (dry spin), discard flow through and collection tube.
19. Add inner columns to nuclease free 1.5ml tubes (pre-warmed to 80^{0}C), incubate at 80^{0}C for 1min with caps open (this will evaporate traces of alcohol, do not exceed 1min).
20. Add 100µl EB (pre-warmed at 80^{0}C) to inner columns, close caps, incubate for 1min at 80^{0}C (do not exceed 1min).
21. Centrifuge columns at 4000rpm (1,180 X G) for 2mins (Fraction A).
22. Add inner columns to new nuclease-free 1.5ml tubes (pre-warmed to 80^{0}C), add 80µl EB (pre-warmed at 80^{o}C) to inner columns, close caps, incubate for 1min at 80^{0}C (do not exceed 1min).
28. Centrifuge columns at 4000rpm (1,180 X G) for 2min (Fraction B).

C. UPDATED GRAM NEGATIVE CELLS PROTOCOL

Up to 1 to 1.5ml O/N culture

1. Centrifuge 1 to 1.5ml of O/N culture at 11000rpm (8,928 X G) for 3mins to pellet the cells, then remove supernatant with a p200 tip and repeat spin with 1ml of 1x PBS
2. Snap-freeze pellets at -80^{0}C for about 5-10mins to produce greater yields.
3. Add 100µl of 20mg/ml lysozyme solution (1.2% triton X-100) to each pellet, resuspending by 5x aspirations (p200 set to 100µl, narrow bore tip) followed by 5secs of vortexing at max speed.
4. Incubate at 37^{0}C for 10mins.
5. During lysozyme incubation make a stock of 300µl of LSDNA, plus 20ml of Proteinase K, plus 20µl of RNase A (Sigma-Aldrich #R6148 is recommended) per sample by vortexing for 10secs at max speed. Stock solution needs to be made fresh right before use and not kept on ice.
6. After lysozyme incubation add 340µl of the stock solution to each sample and mix by 5x aspirations with a p1000 (set to 400µl, narrow bore), followed by 5secs of vortexing at max speed.
7. Incubate at 56^{0}C for 20mins.
8. Add 350µl BS, vortex for 3secs at max speed. The solution will turn cloudy.
9. Add 400µl 75% isopropanol, vortex for 3secs at max speed. The solution will turn clear; if not manually rotate the tubes until it does.
10. Load 650µl to the column using a p1000 wide bore tip.
11. Centrifuge at 8000rpm (4,722 X G) for 1min, discard flow through.
12. Load remaining volume to the column using a p1000 wide bore tip.
13. Centrifuge at 8000rpm (4,722 X G) for 1min, discard flow through.
14. Add 500µl WS to the column.
15. Centrifuge at 8000rpm (4,722 X G) for 1min, discard flow through.
16. Add 500µl 90% ethanol to each column.
17. Centrifuge at 14000rpm (14,462 X G) for 3mins, discard flow through.
18. Centrifuge again at 14000rpm (14,462 X G) for 1min (dry spin), discard flow through and collection tube.
19. Add inner columns to nuclease free 1.5ml tubes (pre-warmed to 80^{0}C), incubate at 80^{0}C for 1min with caps open (this will evaporate traces of alcohol, do not exceed 1min).
20. Add 100µl EB (pre-warmed at 80^{0}C) to inner columns, close caps, incubate for 1min at 80^{0}C (do not exceed 1min).
21. Centrifuge columns at 4000rpm (1,180 X G) for 2mins (Fraction A).
22. Add inner columns to new nuclease-free 1.5ml tubes (pre-warmed to 80^{0}C), add 80µl EB (pre-warmed at 80^{o}C) to inner columns, close caps, incubate for 1min at 80^{0}C (do not exceed 1min).
23. Centrifuge columns at 4000rpm (1,180 X G) for 2min (Fraction B).

D. LYSATE FRAGMENTATION MAMMALIAN PROTOCOL

Up to 3-5 million mammalian cells, or 1ml of whole blood

1. Add 5X blood volume of ice-cold RBC lysis solution (100mM NH_4Cl, 10mM $NaHCO_3$, 0.1mM EDTA, pH 7.3) to up to 1ml of whole blood and vortex to mix thoroughly. ***For all other mammalian cell types pellet the cells. Remove all supernatant, after washing 1x in 1x PBS (do not wash in TE as this could have a negative effect on yield) and go straight to step 9.***
2. Incubate at RT for 5-10mins; vortex 3-5 times during incubation (for samples with volumes that fill more than half of the tube, invert before immediately placing upright onto vortex).
3. Spin at 250 x G for 3min and discard supernatant by gently tipping into waste.
4. Add 2X blood volume of ice-cold RBC lysis solution; vortex gently on lowest setting for 10s.
5. Spin at 250 x G for 3 min.
6. Pipette foam off the top of the supernatant and gently tip the supernatant into the waste; allow each pellet to rest on ice so that the excess supernatant pools.
7. Using a pipette carefully remove excess supernatant; invert tube as you draw the pipette.
8. Keep pellets on ice until use (use as soon as possible, keep on ice no longer than 1hr).
9. Just before use make a stock of 300µl of LSDNA, plus 20µl of Proteinase K, plus 10µl of RNase A (Sigma-Aldrich #R6148 is recommended) per sample by vortexing for 10secs at max speed. Stock solution needs to be made fresh right before use and not kept on ice.
10. Add 330µl of the stock solution to each sample and mix by 5x aspirations with a p1000 (narrow bore tip, set at 400µl) followed by 5secs of vortexing at max speed.
11. Incubate at 56°C for 10mins.
12. Add 350µl BS, vortex for 3secs at max speed. The solution will turn cloudy.
13. Add 400µl 75% isopropanol, vortex for 3secs at max speed. The solution will turn clear; if not manually rotate.
14. Resuspend lysate through a 26G needle (1 ml syringe) 1 to 10x. ***Place needle at bottom of 1.5ml tube, fully extend plunger, and hold until entire sample has been drawn into syringe (~1.5 - 2s). Lift needle slightly from bottom of tube and push plunger down steadily until entire sample has been expelled back into tube (~1 - 1.5s). Repeat with no pauses until total number of required resuspensions are complete.***
15. Use a p1000 and a wide bore tip to load 650µl of the sample to the spin column.
16. Centrifuge at 8000rpm (4,722 x G) for 1min, discard flow through.
17. Use a p1000 and a wide bore tip to load the remaining sample volume to the column.
18. Centrifuge at 8000rpm (4,722 x G) for 1min, discard flow through.
19. Add 500µl WS to the column.
20. Centrifuge at 8000rpm (4,722 x G) for 1min, discard flow through.
21. Add 500µl 90% ethanol to each column.
22. Centrifuge at 14000rpm (14,462 x G) for 3min, discard flow through.
23. Centrifuge again at 14000rpm (14,462 x G) for 1min (dry spin), discard flow through and collection tube.
24. Add inner columns to nuclease free 1.5 ml tubes at RT.
25. Add 100µl EB (pre-warmed at 80°C) to inner columns, close caps, and incubate for 10min at RT.
26. Centrifuge columns at 4000 rpm (1,180 X G) for 5 min to elute.

!!IMPORTANT NOTES!!

- **ADD ETOH** to WS as per IFU.
- **VORTEX** and resuspend as on protocol. Failure to mix properly by vortexing and re-suspension will have a negative effect on DNA integrity and yield.
- **USE RECOMMENDED RNASE A** (Sigma-Aldrich #R6148). Some RNase A solutions could have a negative effect on DNA integrity and yield.
- **FRACTION A** contains HMW-DNA with an average length of about 100kb (Femto Pulse).
- **FRACTION B** contains roughly half the mass of HMW-DNA (compared to Fracton A) and with an average length of about 120kb (Femto Pulse).
- **FOR MOST SEQUENCING USES** pooled fractions generate optimal read length/throughput results (Figure 1).
- **LYSATE FRAGMENTATION NEEDS TO BE OPTIMISED PER USE.** 1x to 4x 26G needle lysate fragmentations are recommended for ONT applications (Figure 2) and 6x to 10x for PacBio applications (Figure 3).

96hrs run	Throughput (Gb)	Reads #	Read N50 (kb)
All data	160.3	12,952,997	38,6
Pass data Q>7	136.5	10,262,206	40,3
100kb+ data Q>7	13.3	108,878	118,8
Top 40x coverage	**109.42**	**2,907,649**	**51,5**

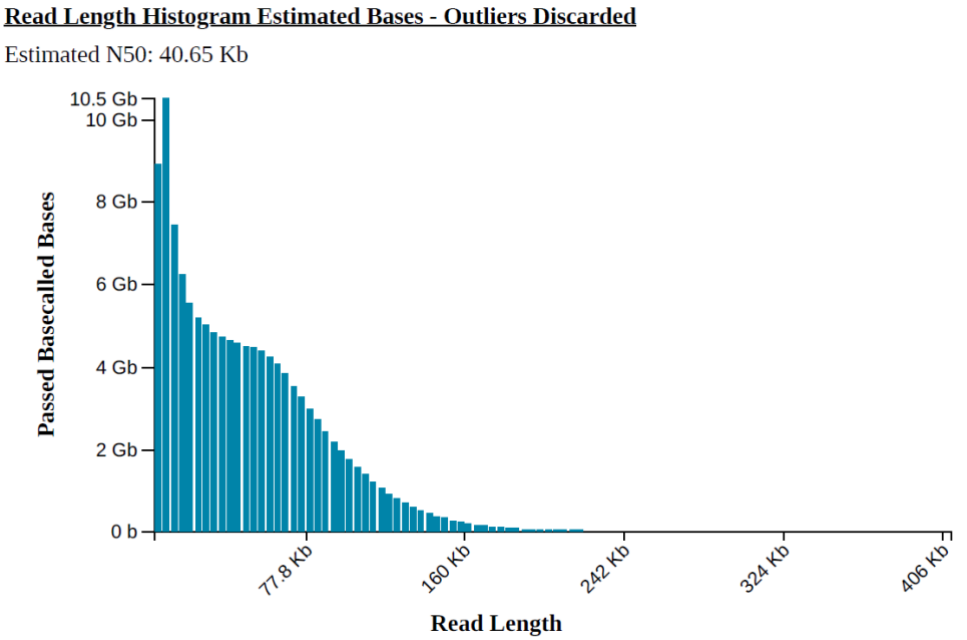

Figure 1. White blood cells from 1ml of horse blood were processed according to updated protocol A.
Fractions A and B were pooled and 48µl of the pool was used on LSK110/PromethION (R.9.4.1 flow cell) **without any size selection post extraction**. 160.3Gb of total data was generated after 96 hrs (3 flushes) 15Gb of which in the last 24hrs. Read N50 for top 40x coverage was 51.5kb.

WBCs from 1ml horse blood (Femto Pulse)

Needling	Average (bp) Rep1	Average (bp) Rep2	ng/µl rep1	ng/µl rep2
1x	34,459	29,032	143.19	96
2x	21,102	24,383	92.22	109.32
3x	22,905	24,190	197.25	151.2
4x	23,873	25,621	113.64	90.6

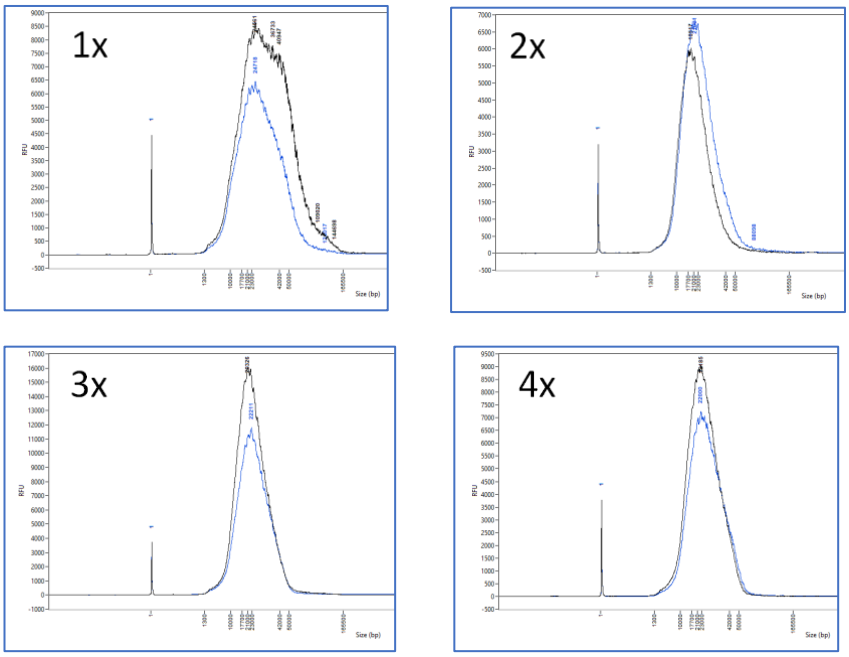

Figure 2. White blood cells from 1ml of horse blood were processed according to lysate fragmentation protocol D.
1x 26G needle re-suspension (protocol step 14) generates extract lengths of about 30-25kb and 2x to 4x extract lengths of about 20 to 25kb.

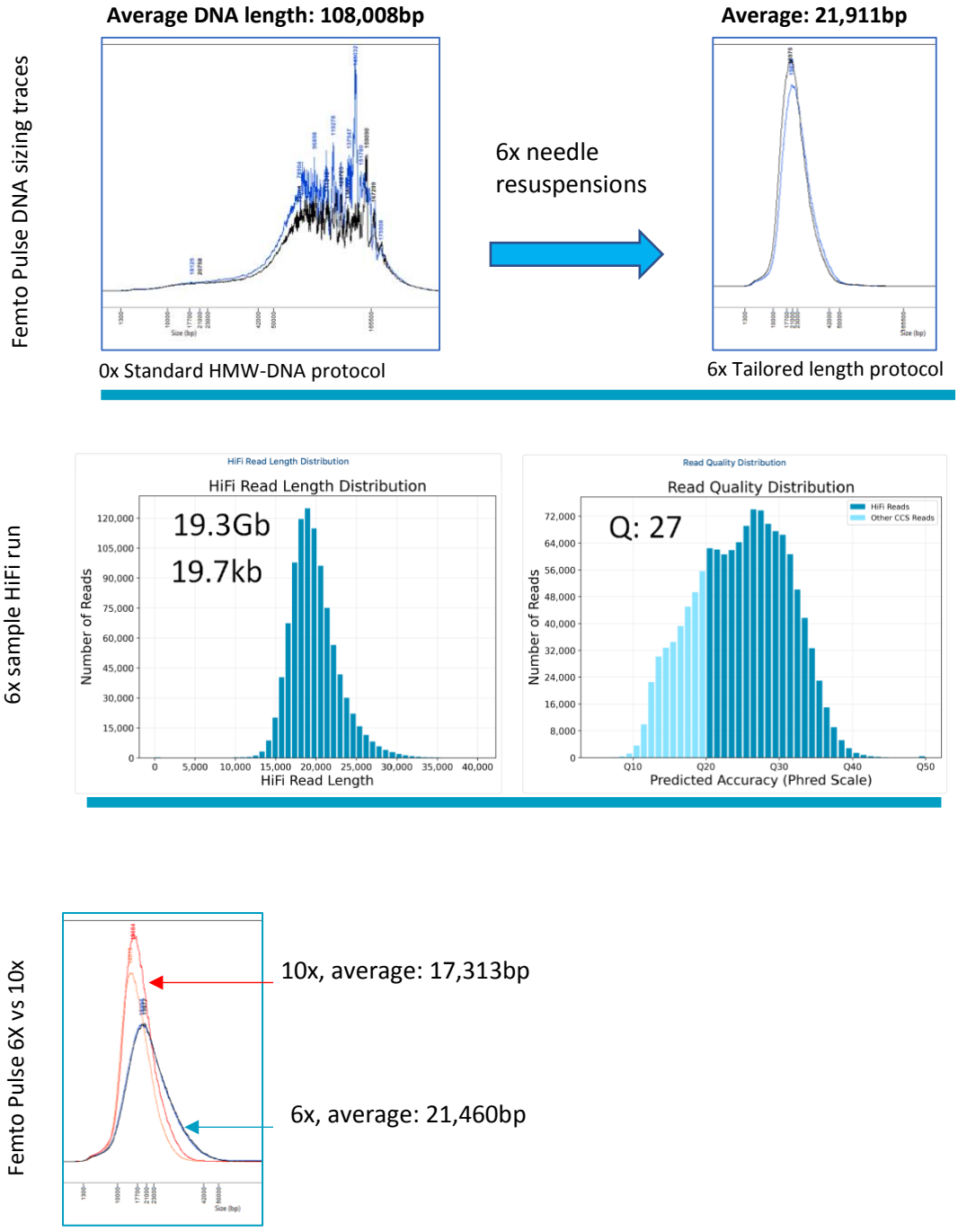

Figure 3. White blood cells from 1ml of horse blood were processed according to lysate fragmentation protocol D.
6x 26G needle re-suspensions (protocol step 14) generates extract lengths of about 20kb (vs 0x Ctrl: 108kb) resulting in 19.3Gb HiFi reads with a mean read of 19.7kb and Q-score: 27 on a single SMRT cell.
10x 26G needle re-suspensions generate extract lengths of about 17kb that could be used to boost HiFi read throughput.