REVOLUGEN

High Molecular Weight
DNA Extraction

Size Selection

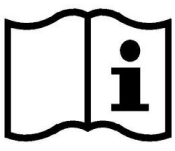
Fire Monkey/Fire Flower (10) kit

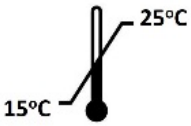
Instructions for Use

For the Extraction of High Molecular Weight DNA from Animal and Bacterial Cells, and Size Selection of Extracted DNA from all Sample Types

For Research Use Only

Not for use in Diagnostic Procedures





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RevoluGen

RevoluGen is a biotech company that provides innovative solutions for the Extraction and Size Selection of High Molecular Weight DNA.

For more information, visit http://www.revolugen.co.uk/ and Twitter: @RevoluGen

Intended Use

The **Fire Monkey** protocol is designed for the extraction of High Molecular Weight (HMW) DNA from bacteria and animal cells. The **Fire Flower** protocol is designed to deplete small DNA fragments from extracted DNA derived from any source. This product may only be used for the DNA procedures set out in the enclosed instructions.

No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

The column is a device that utilises unique technology developed by RevoluGen.

It is intended for use by professional users with training in molecular biological techniques.

The columns are designed for single use only and are disposable with general clinical/lab waste, depending on the sample type.

Kit contents

Fire Monkey/Fire Flower kit	Quantity
Columns in 2ml Collection Tubes	10
Lysis Solution DNA (LSDNA)	10ml
Binding Solution (BS)†	10ml
Wash Solution (WS)‡	5ml
Elution Buffer (EB)	10ml

† This solution contains a chaotropic salt that may form highly reactive compounds with bleach, therefore avoid contact. See page 4 for safety information.

‡ This solution is supplied as a concentrate and needs to have ethanol (96-100%) added according to page 8.

Storage

Columns, Collection tubes and all solutions should be stored in a clean, dry environment at room temperature (15-25^{o}C). Please refer to the expiry date shown on the box for the shelf life.

Equipment and Reagents required but not provided

When working with samples and chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information consult the relevant Safety Data Sheets (SDS), these are also available from http://www.revolugen.co.uk

Equipment required:

- Pipettes and pipette tips (wide bore tips are recommended for column loading)
- Microcentrifuge
- Serological pipettes and pipette gun
- Disposable gloves
- Eppendorfs
- Vortex
- Thermal plate

Reagents required:

- Ethanol (96-100%)
- Isopropanol (98-100%)
- Lysozyme (Lysozyme should be diluted in DNase/RNase-free H_2O [plus 1.2% Triton-X] since some salts could have a negative effect on extract yield and integrity)
- Proteinase K solution (20mg/ml)
- Red blood cell lysis solution (150mM NH_4Cl, 10mM $NaHCO_3$, 0.1mM EDTA, pH7.3)

Optional:

- RNase A. Some RNase A solutions can have a negative effect on extract yield and integrity. It is recommended that you use the Sigma-Aldrich RNase A solution (#R6148).

Quality Control

In accordance with RevoluGen's quality control policy, each lot of Fire Monkey/Fire Flower kits are tested against predetermined specifications to ensure consistent product and quality.

Safety Information

For Research Use Only. Not Intended for use in Diagnostic Procedures.

When working with samples and chemicals, always wear a suitable lab coat, disposable gloves and protective goggles (personal protective equipment). For more information, please consult the appropriate Safety Data Sheets (SDS's) supplied with this product and available from; www.revolugen.co.uk

 **CAUTION: DO NOT add bleach or acidic solutions directly to the sample preparation waste.**

Binding Solution (BS) contains guanidine hydrochloride. Guanidine hydrochloride is a chaotropic salt and can form highly reactive compounds with bleach. If liquid waste is spilt it must be soaked up with absorbent material and cleaned with water and suitable laboratory detergent, followed by 1% (v/v) sodium hypochlorite. Do not allow product to enter the drains.

The following risk and safety phrases apply to components of the Fire Monkey/Fire Flower kit:

Classification according to Regulation (EC) No 1272/2008 [CLP]

LSDNA and WS:	
 Warning	Contain Lithium Chloride
Hazard statements	H315: Causes skin irritation. H319: Causes serious eye irritation.
Precautionary statements	P261: Avoid breathing fumes, mist, vapours and spray. P280: Wear protective gloves, protective clothing and eye protection. P302 + P352: IF ON SKIN: Wash with plenty of water. P305 + P338 + P351: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses if present and easy to do so. Continue rinsing and seek medical advice. P301 + P312: IF SWALLOWED: Rinse mouth. Call a POISON CENTRE/doctor if you feel unwell.
BS:	
 Warning	Contains Guanidine hydrochloride
Hazard statements	H315: Causes skin irritation. H319: Causes serious eye irritation.
Precautionary statements	P261: Avoid breathing fumes, mist, vapours and spray. P280: Wear protective gloves, protective clothing and eye protection. P302 + P352: IF ON SKIN: Wash with plenty of water. P304 + P340: IF INHALED: Remove person to fresh air and keep comfortable for breathing. P305 + P338 + P351: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do so. Continue rinsing and seek medical advice. P301 + P312: IF SWALLOWED: Rinse mouth. Call a POISON CENTRE/doctor if you feel unwell.

Classification according to European Directive 67/548/EEC

LSDNA and WS: harmful, irritant. Risk and safety phrases:§ R22, R36/37/38, S26, S36/37/39.

BS: harmful, irritant. Risk and safety phrases:§ R22, R36/38, S26, S36/39.

§R22: Harmful if swallowed; R36/38: Irritating to eyes and skin; R36/37/38: Irritating to eyes, respiratory system and skin; S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; S36/37/39: Wear suitable protective clothing, gloves and eye/face protection.

Summary

Fire Monkey is a rapid, easy and reliable method for extracting high quality High Molecular Weight (HMW) DNA from animal and bacterial cells within one hour. The extracted DNA can then be used for research purposes. If RNA is present in the sample, it will also be extracted, for the most part, in Fraction A. This allows the researcher to retain all genetic information (DNA+RNA) derived from small quantities of precious samples. A simple DNase or RNase treatment can be employed at the end of the techniques to focus on one type of genetic information (either RNA or DNA).

The product is of high purity with an average strand length which can be 100kb or longer (Figure 1; values stated are subject to original sample quality). In addition to long fragments the Fire Monkey extract carries few of the smaller DNA fragments (under 10kb) which tend to challenge long-read sequencing technologies. It is recommended that 2 fractions are collected. Both fractions will contain HMW DNA, with Fraction A generating a greater overall mass and Fraction B higher average strand lengths due to small fragment depletion during the first elution step (refer to Figure 1). This unique combination of purified long DNA fragments along with very few of the shorter fragments, means that no SPRI-bead based post-extraction size-selection/pre-library process is necessary. The overall result means that N50 reads of ~50kb for animal DNA, and complete assembly with full plasmid recovery for bacterial DNA can be achieved (Figure 2).

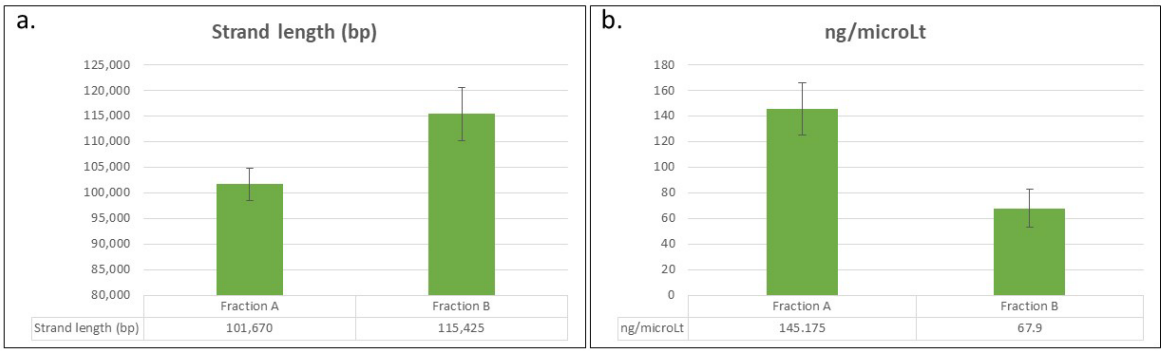
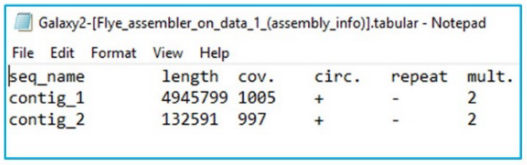


Figure 1. Quadruplicate 1ml horse blood Fire Monkey DNA samples. (a) Femto Pulse analysis (gDNA 165kb protocol) shows that Fraction B contains higher fragment averages than Fraction A. (b) Fraction A contains higher nucleic acid concentration than Fraction B (Nanodrop).

a.

Fire Monkey White Blood Cell (1ml blood) DNA extract sequenced on R9.4.1/LSK109/MinION for 48hrs (NanoPlot)		
Q-score cut-off	≥7	≥11
N50 (bp)	**47,244**	**52,939**
Mean read length (bp)	22,130	27,387
Mean read quality	10.3	11.6
# of reads	363,742	106,805
Total bases	8,049,945,447	2,925,147,551
Top read (bp)	286,934	217,959
# of reads ≥100,000bp	**5,682**	**2,290**

b.



- Flye --asm_coverage set to 50
- 50x coverage with fragments >122kb
- 2x circular contigs at ~1000x coverage (Q7), after one polishing step
- Smaller contig harbours plasmid genes (rep, tra)

Figure 2. (a) A Fire Monkey 1ml horse white blood cell extract generated N50 values of ~50kb and (b) a Fire Monkey 600 million *Escherichia coli* extract enabled a complete assembly with ~1000x coverage of the chromosome (Flye, Q7) and recovery of a ~130kb plasmid (Q7, ~1000x coverage). In both cases 47µl of Fraction B were sequenced for 48hrs according to the ONT LSK109 protocol (R9.4.1, MinION). Due to the Fire Monkey's inbuilt size exclusion aspect no post-extraction/pre-library 0.7x SPRI beads size exclusion step was necessary.

The kit also offers **Fire Flower** which is a stand-alone size-selection protocol. The Fire Flower protocol is used to size select a DNA input sample that has been extracted using any DNA extraction procedure, including Fire Monkey. Fire Flower is extremely simple and rapid. Small DNA fragments (under 10kb) can be depleted from all extracted DNA within 10-15mins (Figure 3).

The Fire Monkey and Fire Flower extracts are also suitable for applications other than long-read sequencing such PCR.

The eluted Nucleic Acid can be used immediately or stored at 4°C for future use.

NanoPlot (LSK109 on MinION, 48hrs)	FF Input	FF Output	Long fragment metrics X increase
Average (bp)	**17,012**	**37,163**	**2.18**
Q-score	10.6	10.7	-
N50 (bp)	**36,919**	**51,959**	**1.4**
Total # of reads	215,077	161,295	-
Total yield (Gb)	3,69	5,99	-
# reads >50kb	**17,460**	**44,917**	**2.57**
# reads >100kb	**1,575**	**3,713**	**2.35**
# reads >150kb	**123**	**284**	**2.3**
Top read	269,583	282,982	-

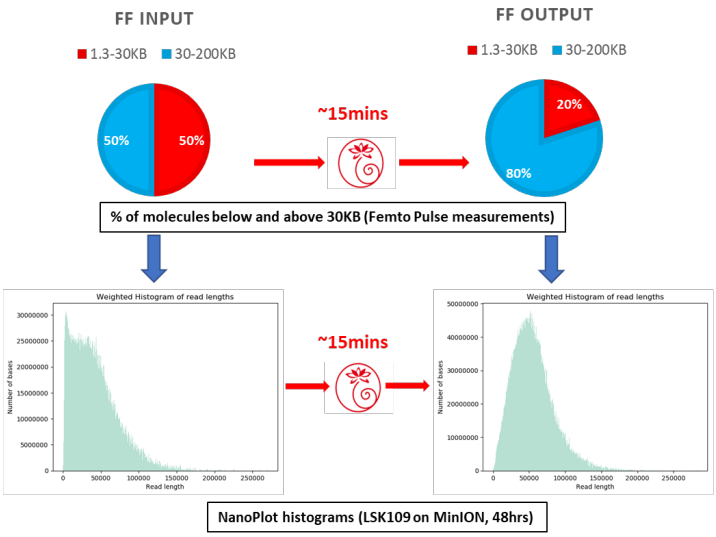


Figure 3. 200µl of a mixture of high and low molecular weight horse blood DNA was used as an input to the Fire Flower (FF) process. After ~15mins the % of molecules above 30kb was increased by 30% (Femto Pulse, gDNA 165kb protocol). Both input and Fire Flower Fraction B were sequenced for 48hrs according to the ONT LSK109 protocol (R9.4.1, MinION). The Fire Flower process resulted in doubling most long-read metrics.

Important points before starting

- When receiving the Fire Monkey/Fire Flower kit please ensure that none of the blister packs have broken seals and that none of the bottles are damaged. If there is any damage, please contact the distributor or RevoluGen. Please consult the 'Safety Information' (page 4) in the case of liquid spillage.
- **DO NOT** add bleach or acidic solutions directly to the sample preparation waste due to the presence of chaotropic agents (see Safety Information for more info).
- We recommend that when using potentially infectious samples that the procedure be carried out in a Class II Microbiological Safety cabinet until the samples are lysed.
- Always use disposable gloves and regularly check that they are not contaminated. If they become contaminated remove immediately and discard.
- We also recommend that filter-tips are used and are for single use to minimise cross-contamination.
- When using the Fire Monkey/Fire Flower kit ensure that the pipette tip does not touch the Column membrane.
- It is recommended to use wide bore tips for column loading.
- Please read Safety Information and SDS before starting and adhere to disposal protocol for waste containing BS, LSDNA and WS. All other lab/clinical waste should be disposed of by autoclaving or incineration.
- Do not re-use the columns.
- Avoid carry over of buffers or solutions not offered with the kit, since this could have a negative effect on both yield and integrity. For example, Lysozyme and RNase A should be diluted in H_2O unless otherwise stated, and cell pellets should carry minimal amounts of buffers such as PBS.
- Overloading the column could have a negative effect on DNA length as seen in Table 1. **Input sample titration is highly recommended.**
- Vortex as indicated in the protocol and make sure that the solution is clear before adding it to the column. **Failure to mix the solutions properly could have a negative effect on both DNA length and yield**.

Escherichia coli (millions)	Fraction A average (bp)	Fraction A ng/µl	Fraction B average (bp)	Fraction B ng/µl
250	103,690	29.7	133,323	17.4
500	100,944	44.5	118,834	30.3
1000	96,867	48.3	93,700	32.7

Table 1. Femto Pulse analysis (gDNA 165kb) of an *Escherichia coli* Fire Monkey titration (plus RNase A) shows decreasing average fragment sizes with increased cell loading. This effect correlates with concentration saturation according to Nanodrop measurements.

Preparation of Solutions

- **Preparation of Wash Solution (WS)**
  Using a pipette gun and serological pipette or measuring cylinder, add 5ml ethanol (96-100%) to the bottle containing 5ml WS concentrate. Mix by gentle agitation and store at room temperature (15-25^{o}C).

Protocols

- Fire Monkey High Molecular Weight DNA Extraction from up to 100µl of **whole blood**

1. **Add up to 100µl of whole blood to a nuclease-free 1.5ml tube**. *Use fresh or previously frozen blood.*
2. **Add 300µl of LSDNA and mix by 5x pipette aspirations** (p1000 set to 400µl, narrow bore filter tip), **then add 20µl of Proteinase K and vortex for 5s at max speed** (up to 3,200rpm).
3. **Incubate at 56°C for 20min and remove from heat source.** *Once completed, raise the temperature to 80°C to pre-warm 2x new 1.5ml tubes per sample and another tube containing sufficient EB (~250µl) to elute two fractions per sample.*
4. **Vortex for 3s at max speed, then add 10µl of RNase A and vortex again for 3s at max speed; incubate at room temperature (RT) for 5min**.
5. **Add 350µl of BS and vortex for 3s at max speed**. Incubate at RT for 10min, then centrifuge at 14,000rpm (14,462 X G) for 15min.
6. **Transfer the supernatant into a new 1.5ml tube and discard the pellet**. *To avoid disturbance of the pellet, gently pour the supernatant into the new tube then use a 200µl wide bore tip to collect the remaining liquid.*
7. **Add 400µl of 75% isopropanol and vortex for 3s at max speed**.
8. **Use a p1000 and a wide bore tip to load 600µl of the sample to the column in the collection tube** (supplied with the kit).
9. **Centrifuge the column in the collection tube at 8,000rpm (4,722 x G) for 1min**, discard flow through.
10. **Use a p1000 and a wide bore tip to load the remaining sample volume to the column in the collection tube.**
11. **Centrifuge the column in the collection tube at 8,000rpm (4,722 x G) for 1min, discard flow through.** If there is liquid remaining in the spin column at this stage, centrifuge at 11,000rpm (8,928 X G) for 1min and discard flow through before continuing.
12. **Add 500µl of WS to the spin column**.
13. **Centrifuge the column in the collection tube at 8,000rpm (4,722 x G) for 1min**, discard flow through.
14. **Add 500µl of 90% ethanol to the spin column**.
15. **Centrifuge the column in the collection tube at 14,000rpm (14,462 x G) for 3min**, discard flow through.
16. **Centrifuge the column in the collection tube again at 14,000rpm (14,462 x G) for 1min** (dry spin), discard flow through and collection tube.
17. **Add the spin column to a nuclease-free 1.5ml collection tube (pre-warmed to 80°C) and incubate at 80°C for 1min with the cap open**. *This will evaporate traces of alcohol, do not exceed 1min.*
18. **Add 100µl EB (pre-warmed at 80°C) to the spin column and incubate at 80°C for 1min with the cap closed**. *Do not exceed 1min.*
19. **Centrifuge the column in the collection tube at 4000rpm (1,180 x G) for 2min to elute: Fraction A.**
20. **Add the spin column to a new nuclease-free 1.5ml collection tube (pre-warmed to 80°C).**

21. **Add 80µl EB (pre-warmed at 80°C) to the spin column and incubate at 80°C for 1min with the cap closed**. *Do not exceed 1min.*
22. **Centrifuge at 4000rpm (1,180 x G) for 2min to elute: Fraction B.**

Overnight elution

As an alternative to the standard elution (steps **17-22**), columns may be incubated overnight with elution buffer to achieve a greater yield of extracted HMW-DNA. Replace steps **17-22** with the following:

17. Add the spin column to a new nuclease-free 1.5ml collection tube at RT.
18. Add 130µl of RT EB to the spin column and incubate overnight at RT with the cap closed.
19. Centrifuge the column in the collection tube at 6,000rpm (2,656 x G) for 2min to elute: Fraction A.
20. Add the spin column to a new nuclease-free 1.5ml collection tube (pre-warmed to 80°C).
21. Add 80µl of EB (pre-warmed to 80°C) to the spin column and incubate at 80°C for 1min with the cap closed.
22. Centrifuge at 4000rpm (1,180 x G) for 2min to elute: Fraction B.

- Fire Monkey High Molecular Weight DNA Extraction from white blood cells using up to 1ml of whole blood as starting material

1. **Use fresh blood or previously frozen blood.**
2. **Briefly vortex aliquots**. *NOTE: White blood cells tend to sediment very quickly; invert the tube of whole blood several times. If frozen blood is used it should be thawed only once – DO NOT store at room temperature or 4°C.*
3. **Add 5X blood volume of ice-cold 1x RBC lysis buffer (150mM NH_4Cl, 10mM $NaHCO_3$, 0.1mM EDTA, pH7.3) to each sample** (e.g to 1ml of blood add 5ml of 1x RBC lysis buffer); **vortex for ~5s at max speed** (up to 3,200rpm) **to mix thoroughly.**
4. **Incubate at room temperature (RT) for 5min; vortex gently 3-5 times during incubation**.
5. **Centrifuge at 250G for 3min** and discard supernatant by gently tipping into a waste container.
6. **Add 2X blood volume of ice-cold 1x RBC lysis buffer**; vortex gently on lowest setting for 10s.
7. **Centrifuge at 250G for 3min**.
8. **Pipette the foam off the top of the supernatant and gently tip the supernatant into a waste container; allow each pellet to rest in ice so that the excess supernatant pools**.
9. **Use a pipette to carefully remove excess supernatant**; invert tube as you draw the pipette.
10. **Keep pellets on ice until use** (use as soon as possible, keep on ice no longer than 1hr).
11. **Add 20µl of Proteinase K into the bottom of a 1.5ml tube. Add 300µl of LSDNA to the cell pellet and resuspend by 5x pipette aspirations, then transfer into the 1.5ml tube containing Proteinase K and vortex for 5s at max speed.**
12. **Incubate at 56°C for 10min and remove from heat source**. *Once completed, raise the temperature to 80°C to pre-warm 2x new 1.5ml tubes per sample and another tube containing sufficient EB (~250µl) to elute two fractions per sample.*
13. **Add 10µl of RNase A and vortex for 3s at max speed, then incubate at RT for 5min**.
14. **Add 350µl of BS and vortex for 3s at max speed**. *The solution will turn cloudy.*
15. **Add 400µl of 75% isopropanol and vortex for 3s at max speed**. *The solution will turn clear – if not, manually invert the tube until it does.*
16. **Use a p1000 and a wide bore tip to load 600µl of the sample to the column in collection tube** (supplied with the kit).
17. **Centrifuge the column in the collection tube at 8,000rpm (4,722 x G) for 1min**, discard flow through.
18. **Use a p1000 and a wide bore tip to load the remaining sample volume to the column in the collection tube**.
19. **Centrifuge the column in the collection tube at 8,000rpm (4,722 x G) for 1min**, discard flow through. If there is liquid remaining in the spin column at this stage, centrifuge at 11,000rpm (8,928 X G) for 1min and discard flow through before continuing.
20. **Add 500µl of WS to the column**.
21. **Centrifuge the column in the collection tube at 8,000rpm (4,722 x G) for 1min**, discard flow through.
22. **Add 500µl of 90% ethanol to the spin column**.

23. **Centrifuge the column in the collection tube at 14,000rpm (14,462 x G) for 3min**, discard flow through.
24. **Centrifuge the column in the collection tube again at 14,000rpm (14,462 x G) for 1min** (dry spin), discard flow through and collection tube.
25. **Add the spin column to a nuclease-free 1.5ml collection tube (pre-warmed to 80°C) and incubate at 80°C for 1min with the cap open**. *This will evaporate traces of alcohol, do not exceed 1min.*
26. **Add 100µl EB (pre-warmed to 80°C) to the spin column and incubate at 80°C for 1min with the cap closed.** *Do not exceed 1min.*
27. **Centrifuge the column in the collection tube at 4,000rpm (1,180 x G) for 2min to elute: Fraction A.**
28. **Add the spin column to a new nuclease-free 1.5ml collection tube (pre-warmed to 80°C)**.
29. **Add 80µl EB (pre-warmed to 80°C) to the spin column and incubate at 80°C for 1min with the cap closed**. *Do not exceed 1min.*
30. **Centrifuge at 4,000rpm (1,180 x G) for 2min to elute: Fraction B.**

Overnight elution

As an alternative to the standard elution (steps **25-30**), columns may be incubated overnight with elution buffer at Room Temperature to achieve a greater yield of extracted HMW-DNA. Replace steps **25-30** with the following:

25. Add the spin column to a new nuclease-free 1.5ml collection tube at RT.
26. Add 130µl of RT EB to the spin column and incubate overnight at RT with the cap closed.
27. Centrifuge the column in the collection tube at 6,000rpm (2,656 x G) for 2min to elute: Fraction A.
28. Add the spin column to a new nuclease-free 1.5ml collection tube (pre-warmed to 80°C).
29. Add 80µl of EB (pre-warmed to 80°C) to the spin column and incubate at 80°C for 1min with the cap closed. *Do not exceed 1min.*
30. Centrifuge at 4,000rpm (1,180 x G) for 2min to elute: Fraction B.

- Fire Monkey High Molecular Weight DNA Extraction from white blood cells using 1-5ml of whole blood as starting material

1. **Use fresh blood or previously frozen blood.**
2. **Briefly vortex aliquots**. *NOTE: White blood cells tend to sediment very quickly; invert the tube of whole blood several times. If frozen blood is used it should be thawed only once – DO NOT store at room temperature or 4°C.*
3. **Add 5X blood volume of ice-cold 1x RBC lysis buffer (150mM NH_4Cl, 10mM $NaHCO_3$, 0.1mM EDTA, pH7.3) to each sample** (e.g to 5ml of blood add 25ml of 1x RBC lysis buffer); **vortex for ~5s at max speed** (up to 3,200rpm) **to mix thoroughly.**
4. **Incubate at room temperature (RT) for 5min; vortex gently 3-5 times during incubation**.
5. **Centrifuge at 250G for 3min** and discard supernatant by gently tipping into a waste container.
6. **Add 2X blood volume of ice-cold 1x RBC lysis buffer**; vortex gently on lowest setting for 10s.
7. **Centrifuge at 250G for 3min**.
8. **Pipette the foam off the top of the supernatant and gently tip the supernatant into a waste container; allow each pellet to rest in ice so that the excess supernatant pools**.
9. **Use a pipette to carefully remove excess supernatant**; invert tube as you draw the pipette.
10. **Keep pellets on ice until use** (use as soon as possible, keep on ice no longer than 1hr).
11. **Add 20µl of Proteinase K into the bottom of a 1.5ml tube. Add 300µl of LSDNA to the cell pellet and resuspend by 5x pipette aspirations** (p1000 set to 400µl, narrow bore filter tip), **then transfer into the 1.5ml tube containing Proteinase K and vortex for 5s at max speed.**
12. **Resuspend the sample by 6x pipette aspirations** (p200 set to 200µl, narrow bore filter tip)**, then incubate at 56°C for 10min.** *Once completed, raise the temperature to 80°C to pre-warm 2x new 1.5ml tubes per sample and another tube containing sufficient EB (~250µl) to elute two fractions per sample.*
13. **Remove from the heat source and vortex for 3s at max speed, then add 10µl of RNase A and vortex again for 3s at max speed; incubate at room temperature (RT) for 5min**.
14. **Add 350µl of BS and vortex for 3s at max speed**. **Incubate at RT for 10min, then centrifuge at 14,000rpm (14,462 X G) for 15min.**
15. **Transfer the supernatant into a new 1.5ml tube and discard the pellet**. *To avoid disturbance of the pellet, carefully remove the liquid using a 200µl wide bore tip.*
16. **Add 400µl of 75% isopropanol and vortex for 3s at max speed**.
17. **Use a p1000 and a wide bore tip to load 600µl of the sample to the column in the collection tube** (supplied with the kit).
18. **Centrifuge the column in the collection tube at 8,000rpm (4,722 x G) for 1min**, discard flow through.
19. **Use a p1000 and a wide bore tip to load the remaining sample volume to the column in the collection tube.**
20. **Centrifuge the column in the collection tube at 8,000rpm (4,722 x G) for 1min, discard flow through.** If there is liquid remaining in the spin column at this stage, centrifuge at 11,000rpm (8,928 X G) for 1min and discard flow through before continuing.
21. **Add 500µl of WS to the spin column**.

22. **Centrifuge the column in the collection tube at 8,000rpm (4,722 x G) for 1min**, discard flow through.
23. **Add 500µl of 90% ethanol to the spin column**.
24. **Centrifuge the column in the collection tube at 14,000rpm (14,462 x G) for 3min**, discard flow through.
25. **Centrifuge the column in the collection tube again at 14,000rpm (14,462 x G) for 1min** (dry spin), discard flow through and collection tube.
26. **Add the spin column to a nuclease-free 1.5ml collection tube (pre-warmed to 80°C) and incubate at 80°C for 1min with the cap open**. *This will evaporate traces of alcohol, do not exceed 1min.*
27. **Add 100µl EB (pre-warmed at 80°C) to the spin column and incubate at 80°C for 1min with the cap closed**. *Do not exceed 1min.*
28. **Centrifuge the column in the collection tube at 4000rpm (1,180 x G) for 2min to elute: Fraction A.**
29. **Add the spin column to a new nuclease-free 1.5ml collection tube (pre-warmed to 80°C).**
30. **Add 80µl EB (pre-warmed at 80°C) to the spin column and incubate at 80°C for 1min with the cap closed**. *Do not exceed 1min.*
31. **Centrifuge at 4000rpm (1,180 x G) for 2min to elute: Fraction B.**

Overnight elution

As an alternative to the standard elution (steps **26-31**), columns may be incubated overnight with elution buffer to achieve a greater yield of extracted HMW-DNA. Replace steps **26-31** with the following:

26. Add the spin column to a new nuclease-free 1.5ml collection tube at RT.
27. Add 130µl of RT EB to the spin column and incubate overnight at RT with the cap closed.
28. Centrifuge the column in the collection tube at 6,000rpm (2,656 x G) for 2min to elute: Fraction A.
29. Add the spin column to a new nuclease-free 1.5ml collection tube (pre-warmed to 80°C).
30. Add 80µl of EB (pre-warmed to 80°C) to the spin column and incubate at 80°C for 1min with the cap closed.
31. Centrifuge at 4000rpm (1,180 x G) for 2min to elute: Fraction B.

- Fire Monkey High Molecular Weight DNA Extraction from Mammalian cells (up to 5 million cells)

1. **Pellet cells**. Remove all supernatant after 1x wash in 1x PBS *(do not wash in TE as this could have a negative effect on yield).*
2. **Add 20µl of Proteinase K into the bottom of a 1.5ml tube. Add 300µl of LSDNA to the cell pellet and resuspend by 5x pipette aspirations** (p1000 set to 400µl, narrow bore filter tip), **then transfer into the 1.5ml tube containing Proteinase K and vortex for 5s at max speed** (up to 3,200rpm).
3. **Incubate at 56°C for 10min and remove from heat source**. *Once completed, raise the temperature to 80°C to pre-warm 2x new 1.5ml tubes per sample and another tube containing sufficient EB (~250µl) to elute two fractions per sample.*
4. **Add 10µl of RNase A and vortex for 3s at max speed** (up to 3,200rpm)**, then incubate at room temperature (RT) for 5min**.
5. **Add 350µl of BS and vortex for 3s at max speed**. *The solution will turn cloudy.*
6. **Add 400µl of 75% isopropanol and vortex for 3s at max speed**. *The solution will turn clear – if not, manually invert the tube until it does.*
7. **Use a p1000 and a wide bore tip to load 600µl of the sample to the column in the collection tube** (supplied with the kit).
8. **Centrifuge the column in the collection tube at 8,000rpm (4,722 x G) for 1min,** discard flow through.
9. **Use a p1000 and a wide bore tip to load the remaining sample volume to the column in the collection tube.**
10. **Centrifuge the column in the collection tube at 8,000rpm (4,722 x G) for 1min,** discard flow through**.** If there is liquid remaining in the spin column at this stage, centrifuge at 11,000rpm (8,928 X G) for 1min and discard flow through before continuing.
11. **Add 500µl of WS to the column in the collection tube**.
12. **Centrifuge the column in the collection tube at 8000rpm (4,722 x G) for 1min**, discard flow through.
13. **Add 500µl of 90% ethanol to the column in the collection tube**.
14. **Centrifuge the column in the collection tube at 14,000rpm (14,462 x G) for 3min**, discard flow through.
15. **Centrifuge the column in the collection tube again at 14000rpm (14,462 x G) for 1min** (dry spin), discard flow through and collection tube.
16. **Add the spin column to a nuclease-free 1.5ml collection tube (pre-warmed to 80°C) and incubate at 80°C for 1min with the cap open**. *This will evaporate traces of alcohol, do not exceed 1min.*
17. **Add 100µl EB (pre-warmed to 80°C) to the spin column and incubate at 80°C for 1min with the cap closed**. *Do not exceed 1min.*
18. **Centrifuge the column in the collection tube at 4,000rpm (1,180 x G) for 2min to elute: Fraction A.**
19. **Add the spin column to a new nuclease-free 1.5ml tube (pre-warmed to 80°C).**

20. **Add 80µl EB (pre-warmed to 80°C) to the spin column and incubate at 80°C for 1min with the cap closed.** *Do not exceed 1min.*
21. **Centrifuge at 4,000rpm (1,180 x G) for 2min to elute: Fraction B.**

Overnight elution

As an alternative to the standard elution (steps **16-21**), columns may be incubated overnight at Room Temperature with elution buffer to achieve a greater yield of extracted HMW-DNA. Replace steps **16-21** with the following:

16. Add the spin column to a new nuclease-free 1.5ml collection tube at RT.
17. Add 130µl of RT EB to the spin column and incubate overnight at RT with the cap closed.
18. Centrifuge the column in the collection tube at 6,000rpm (2,656 x G) for 2min to elute: Fraction A.
19. Add the spin column to a new nuclease-free 1.5ml collection tube (pre-warmed to 80°C).
20. Add 80µl of EB (pre-warmed to 80°C) to the spin column and incubate at 80°C for 1min with the cap closed.
21. Centrifuge at 4000rpm (1,180 x G) for 2min to elute: Fraction B.

- Fire Monkey High Molecular Weight DNA Extraction from Gram Negative bacteria up to 1ml of an overnight culture (no more than 1 billion cells)

1. **Pellet cells by centrifuging 1ml aliquots of overnight culture at 11,000rpm (8,928 x G) for 3min, then remove supernatant. Pellets can be washed in 1x PBS (do not wash in TE as this could have a negative effect on yield).** *Use cell pellets immediately or store at -20°C (defrost at room temperature (RT) for 5-10min before use).*
2. **Prepare a 20mg/ml lysozyme solution with STET buffer: 8% sucrose, 50mM Tris-HCl (pH8), 50mM EDTA (pH8), 5% Triton X-100**. *Lysozyme solution should be prepared immediately before use.*
3. **Add 100µl of lysozyme solution to the cell pellet and resuspend by 10x pipette aspirations** (p200 set to 100µl, narrow bore filter tip), **then vortex for 5s at max speed** (up to 3,200rpm). *Note: 10 secs for hard to lyse cells such as S.enteritidis*.
4. **Incubate at 37°C for 10min**.
5. **Remove from the heat source. Add 300µl of LSDNA and mix by 5x pipette aspirations** (10x for hard to lyse cells such as *S. enteritidis*; p1000 set to 400µl, narrow bore filter tip), **then add 20µl of Proteinase K and vortex for 5s at max speed.**
6. **Incubate at 56°C for 20min and remove from heat source.** *Once completed, raise the temperature to 80°C to pre-warm 2x new 1.5ml tubes per sample and another tube containing sufficient EB (~250µl) to elute two fractions per sample.*
7. **Add 20µl of RNase A and vortex for 3s at max speed, then incubate at room temperature (RT) for 5min**.
8. **Add 350µl of BS and vortex for 3s at max speed.** *The solution will turn cloudy.*
9. **Add 400µl of 75% isopropanol and vortex for 3s at max speed.** *The solution will turn clear – if not, manually invert the tube until it does.*
10. **Use a p1000 and a wide bore tip to load 600µl of the sample to the column in the collection tube** (supplied with the kit).
11. **Centrifuge the column in the collection tube at 8,000rpm (4,722 x G) for 1min**, discard flow through.
12. **Use a p1000 and a wide bore tip to load the remaining sample volume to the column in the collection tube**.
13. **Centrifuge the column in the collection tube at 8,000rpm (4,722 x G) for 1min,** discard flow through. If there is liquid remaining in the spin column at this stage, centrifuge at 11,000rpm (8,928 X G) for 1min and discard flow through before continuing.
14. **Add 500µl of WS to the column in the collection tube.**
15. **Centrifuge the column in the collection tube at 8,000rpm (4,722 x G) for 1min,** discard flow through.
16. **Add 500µl of 90% ethanol to the column in the collection tube.**
17. **Centrifuge the column in the collection tube at 14,000rpm (14,462 x G) for 3min,** discard flow through.
18. **Centrifuge the column in the collection tube again at 14,000rpm (14,462 x G) for 1min** (dry spin), discard flow through and collection tube.

19. **Add the spin column to a nuclease-free 1.5ml collection tube (pre-warmed to 80°C) and incubate at 80°C for 1min with the cap open**. *This will evaporate traces of alcohol, do not exceed 1min.*
20. **Add 100µl EB (pre-warmed at 80°C) to the spin column in the collection tube and incubate at 80°C for 1min with the cap closed**. *Do not exceed 1min.*
21. **Centrifuge the column in the collection tube at 4,000rpm (1,180 x G) for 2min to elute: Fraction A.**
22. **Add the spin column to a new nuclease-free 1.5ml collection tube (pre-warmed to 80°C).**
23. **Add 80µl EB (pre-warmed at 80°C) to the spin column in the collection tube and incubate at 80°C for 1min with the cap closed.** *Do not exceed 1min.*
24. **Centrifuge at 4,000rpm (1,180 x G) for 2min to elute: Fraction B.**

Overnight elution

As an alternative to the standard elution (steps **19-24**), columns may be incubated overnight with elution buffer to achieve a greater yield of extracted HMW-DNA. Replace steps **19-24** with the following:

19. Add the spin column to a new nuclease-free 1.5ml collection tube at RT.
20. Add 130µl of RT EB to the spin column in the collection tube and incubate overnight at RT with the cap closed.
21. Centrifuge the column at 6,000rpm (2,656 x G) for 2min to elute: Fraction A.
22. Add the spin column to a new nuclease-free 1.5ml collection tube (pre-warmed to 80°C).
23. Add 80µl of EB (pre-warmed to 80°C) to the spin column and incubate at 80°C for 1min with the cap closed.
24. Centrifuge at 4000rpm (1,180 x G) for 2min to elute: Fraction B.

- Fire Monkey High Molecular Weight DNA Extraction from Gram Positive bacteria up to 1ml of an overnight culture (no more than 1 billion cells)

1. **Pellet cells by centrifuging 1ml aliquots of overnight culture at 11,000rpm (8,928 x G) for 3min, then remove supernatant. Pellets can be washed in 1x PBS (do not wash in TE as this could have a negative effect on yield).** *Use cell pellets immediately or store at -20°C – defrost at RT for 5-10min before use.*
2. **Prepare a 20mg/ml lysozyme solution with STET buffer: 8% sucrose, 50mM Tris-HCl (pH 8), 50mM EDTA (pH 8), 5% Triton X-100.** *Lysozyme solution should be prepared immediately before use.*
3. **Add 100µl of lysozyme solution to the cell pellet and resuspend by 10x pipette aspirations** (p200 set to 100µl, narrow bore filter tip), **then vortex for 10s at max speed** (up to 3,200rpm).
4. **Incubate at 37°C for 30min.**
5. **Remove from the heat source. Add 300µl of LSDNA and mix by 10x pipette aspirations** (p1000 set to 400µl narrow bore filter tip), **then add 20µl of Proteinase K and vortex for 5s at max speed.**
6. **Incubate at 56°C for 20min and remove from heat source.** *Once completed, raise the temperature to 80°C to pre-warm 2x new 1.5ml tubes per sample and another tube containing sufficient EB (~250µl) to elute two fractions per sample.*
7. **Add 20µl of RNase A and vortex for 3s at max speed, then incubate at room temperature (RT) for 5min**.
8. **Add 350µl of BS and vortex for 3s at max speed.** *The solution will turn cloudy.*
9. **Add 400µl of 75% isopropanol and vortex for 3s at max speed.** *The solution will turn clear – if not, manually invert the tube until it does.*
10. **Use a p1000 and a wide bore tip to load 600µl of the sample to the column in the collection tube** (supplied with the kit).
11. **Centrifuge the column in the collection tube at 8,000rpm (4,722 x G) for 1min,** discard flow through.
12. **Use a p1000 and a wide bore tip to load the remaining sample volume to the column in the collection tube.**
13. **Centrifuge the column in the collection tube at 8,000rpm (4,722 x G) for 1min**, discard flow through. If there is liquid remaining in the spin column at this stage, centrifuge at 11,000rpm (8,928 X G) for 1min and discard flow through before continuing.
14. **Add 500µl of WS to the column in the collection tube**.
15. **Centrifuge the column in the collection tube at 8,000rpm (4,722 x G) for 1min**, discard flow through.
16. **Add 500µl of 90% ethanol to the column in the collection tube**.
17. **Centrifuge the column in the collection tube at 14,000rpm (14,462 x G) for 3min**, discard flow through.
18. **Centrifuge the column in the collection tube again at 14,000rpm (14,462 x G) for 1min** (dry spin), discard flow through and collection tube.
19. **Add the spin column to a nuclease-free 1.5ml collection tube (pre-warmed to 80°C) and incubate at 80°C for 1min with the cap open.** *This will evaporate traces of alcohol, do not exceed 1min.*

20. **Add 100µl EB (pre-warmed at 80°C) to the spin column and incubate at 80°C for 1min with the cap closed.** *Do not exceed 1min.*
21. **Centrifuge the column in the collection tube at 4,000rpm (1,180 x G) for 2min to elute: Fraction A.**
22. **Add the spin column to a new nuclease-free 1.5ml collection tube (pre-warmed to 80°C).**
23. **Add 80µl EB (pre-warmed at 80°C) to the spin column in the collection tube and incubate at 80°C for 1min with the cap closed.** *Do not exceed 1min.*
24. **Centrifuge at 4,000rpm (1,180 x G) for 2min to elute: Fraction B**.

Overnight elution

As an alternative to the standard elution (steps **19-24**), columns may be incubated overnight with elution buffer to achieve a greater yield of extracted HMW-DNA. Replace steps **19-24** with the following:

19. Add the spin column to a new nuclease-free 1.5ml collection tube at RT.
20. Add 130µl of RT EB to the spin column and incubate overnight at RT with the cap closed.
21. Centrifuge the column in the collection tube at 6,000rpm (2,656 x G) for 2min to elute: Fraction A.
22. Add the spin column to a new nuclease-free 1.5ml collection tube (pre-warmed to 80°C).
23. Add 80µl of EB (pre-warmed to 80°C) to the spin column and incubate at 80°C for 1min with the cap closed.
24. Centrifuge at 4000rpm (1,180 x G) for 2min to elute: Fraction B.

- Fire Flower Size Selection

1. **Pre-mix LSDNA/BS/75% isopropanol at a 2/3.5/4 ratio (200/350/400µl per sample) by vortexing.** Pre-mixed solution should be prepared just before use and the solutions used should be added in the following order:
    a. **LSDNA**
    b. **BS** *A white precipitate might form, which will be dissolved after the addition of isopropanol.*
    c. **75% isopropanol**
    The final pre-mixed solution should be clear.
2. **Use wide bore tips to add up to 200µl of extracted DNA to 900µl of pre-mixed solutions and mix by inverting the tube 2-3 times.**
3. **Use wide bore tips to transfer 600µl to the spin column.**
4. **Centrifuge at 4,000rpm (1,180 X G) for 1min.** Discard the flow-through.
5. **Use wide bore tips to transfer the remainder of the sample to the spin column.**
6. **Centrifuge at 4,000rpm (1,180 X G) for 1min.** Discard the flow-through.
7. **Add 500µl of WS to the spin column and centrifuge at 8,000rpm (4,722 X G) for 1 min.** Discard the flow-through.
8. **Add 500µl of 90% ethanol to the spin column and centrifuge at 14,000rpm (14,462 X G) for 3 mins.** Discard the flow-through.
9. **Centrifuge the spin column at 14,000rpm (14,462 X G) for 1 min.** Discard the flow-through.
10. **Transfer the column to a new nuclease-free 1.5ml collection tube.**
11. **Add 100µl of EB to the spin column and incubate at room temperature (RT) for 1 min.** ***DO NOT*** *elute in a volume smaller than 100µl as this could have a negative effect on DNA integrity.*
12. **Elute at 4,000rpm (1,180 X G) for 2mins: Fraction A.** *This fraction contains the smaller DNA fragments and* ***IS NOT RECOMMENDED*** *to be used for downstream sequencing applications.* ***DO NOT*** *re-load the eluate to re-elute in a more concentrated format as this could have a negative effect on DNA integrity.*
13. **Transfer the column to a new nuclease-free 1.5ml collection tube.**
14. **Add 80µl of EB to the spin column and incubate at RT for 1 min.** ***DO NOT*** *elute in a volume smaller than 80µl as this could have a negative effect on DNA integrity.*
15. **Elute at 4,000rpm (1,180 X G) for 2 mins: Fraction B.** This fraction **IS RECOMMENDED** to be used for downstream sequencing applications.

Please note that the High Molecular Weight DNA tends to settle at the bottom of the eluate in the base of the tube.

Trouble Shooting Guide

Question	Solution
How do I pellet the cells?	Use standard protocols depending on cell type. Please ensure that after you wash the pellet all supernatant is removed, since salt carry over could negatively affect yield and DNA strand length. Similarly, do not wash in TE. It is recommended that pelleting is performed with 1x PBS.
How long should I vortex the sample?	The Fire Monkey protocol requires several vortexing steps. Vortex as indicated in the protocols. If vortexing is omitted this will have a negative effect on DNA strand length and yield. We recommend that you avoid excessive vortexing as this could negatively affect nucleic acid integrity.
A white precipitate has formed after the addition of BS, what should I do?	After the addition of alcohol any white precipitates should solubilise. If not, invert manually until clear.
How can I achieve maximal DNA yield?	Follow the overnight elution process for your particular sample type.
What is the maximal starting material I can use for the Fire Monkey Protocol?	Overloading the column should be avoided. In general, loading more than $5x10^6$ mammalian cells, or $1x10^9$ of bacterial cells for the Fire Monkey protocol can have a negative effect on yield and DNA strand length. We recommend performing a loading titration experiment for each particular sample type.
After loading, a small volume of lysed material has remained in the column, what should I do?	This means that the column was overloaded. Spin the column again for 1 minute at 11,000rpm (4,722 x G) as indicated in the protocol.
Can I elute at different volumes? Can I elute in water?	The lowest recommended elution volumes for Fire Monkey and Fire Flower are 100 and 80µl respectively. It is not recommended to elute at lower volumes or re-load the eluate as will have a negative effect on DNA integrity. Both EB or DNase and RNase-free water can be used, however for long term storage, especially for long DNA strands, we recommend the use of buffer EB.
Gram positive protocols generate low yields, how can I increase the yield?	Lysozyme solution should be freshly prepared prior to extraction. Lysozyme concentration and/or incubation time might require optimization. Replace lysozyme with lysostaphin for *Staphylococcus aureus* use.
The solutions were left at temperatures lower than the recommended 15 to 25°C, what should I do?	At relatively low temperatures the LSDNA solution might form precipitates that could affect nucleic acid binding to the column. Incubate the solution at 56°C until the precipitates have fully dissolved.
A small white precipitate appears at the bottom of the elution tube.	This is due to a small amount of silica fibres which do not cause a problem for sequencing. However, the eluate could be spun down and the supernatant can be used for any application.
Low DNA yield from white blood cells.	White blood cells tend to sediment very quickly during aliquoting of the white blood therefore the whole blood container should be inverted several times so that the blood is mixed every 2-3 aliquots. In addition, if blood is not used from fresh, it should be frozen and thawed only once before use (DO NOT store at room temperature or 4°C).
What is the minimal starting material I can use for the Fire Flower protocols?	It is recommended that at least 3µg of input DNA is used.

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