REVOLUGEN

High Molecular Weight
DNA Extraction

Size Selection

Fire Monkey/Fire Flower (50) 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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Contents

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	50
Lysis Solution DNA (LSDNA)	17ml
Binding Solution (BS)†	20ml
Wash Solution (WS)‡	14ml
Elution Buffer (EB)	20ml

† This solution contains a chaotropic salt that may form highly reactive compounds with bleach, therefore avoid contact. See page 5 for safety information.

‡ This solution is supplied as a concentrate and needs to have ethanol (96-100%) added according to page 13.

Storage

Columns, Collection tubes and all solutions should be stored in a clean, dry environment at room temperature (15-25^{0}C) and are stable for 1 year. Please refer to the expiry date shown on the box.

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)

Reagents required (optional):

- RNase A. Some RNase A solutions can have a negative effect on extract yield and integrity. It is recommended that RNase A is diluted in DNase-free H_2O. Alternatively, the Sigma-Aldrich RNase A solutions (#R6148) can be used.

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.

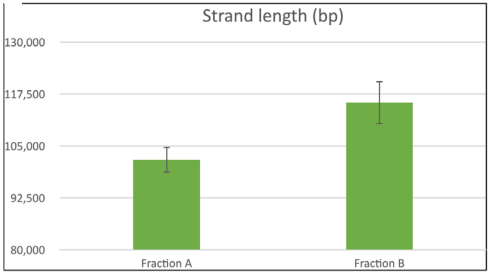
Summary

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). Additionally, RNase A treatment can be applied during Proteinase K lysis to remove any RNA.

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).

a.



b.

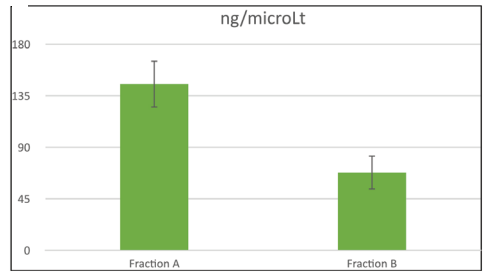


Figure 1. Quadruplicate 1ml horse blood Fire Monkey DNA samples. (a) Femto Pulse analysis (gDNA 165kb protocol) shows that Fraction B contains higher fragment averages than Fraction A. (b) Fraction A contains higher nucleic acid concentration than Fraction B (Nanodrop).

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.

Galaxy2-[Flye_assembler_on_data_1_(assembly_info)].tabular - Notepad

File Edit Format View Help

seq_name	length	cov.	circ.	repeat	mult.
contig_1	4945799	1005	+	-	2
contig_2	132591	997	+	-	2

- 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 *Excherichia 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 bead 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 rapid and simple. 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	-

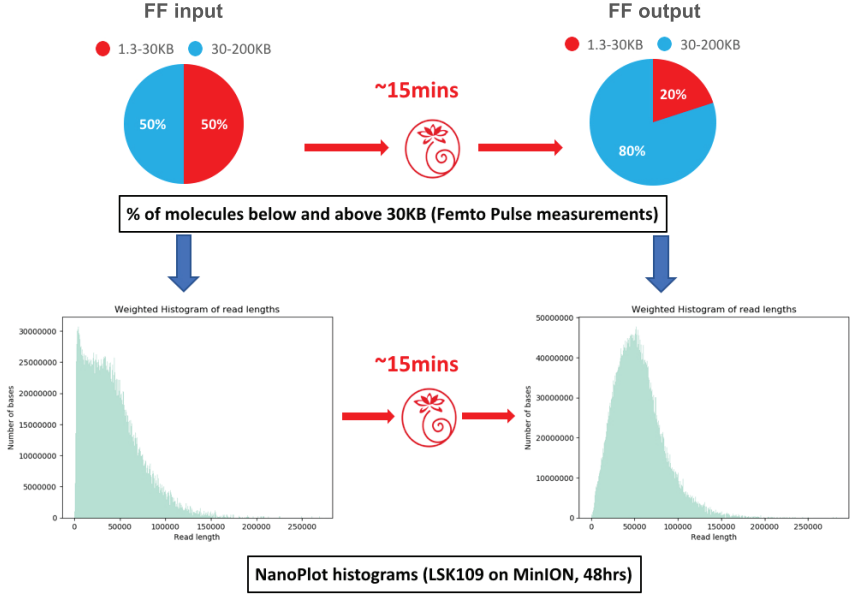


Figure 3. 200µl of a mixture of high and low molecular weight horse blood DNA was used as an input to the Fire Flower (FF) process. After ~15 mins the % of molecules above 30kb was increased by 30% (Femto Pulse, gDNA 165kb protocol). Both input and Fire Flower Fraction B were sequenced for 48hrs according 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 5) 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 on the protocol for a few seconds 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 14ml ethanol (96-100%) to the bottle containing 14ml WS concentrate. Mix by gentle agitation and store at room temperature (15-25^{0}C).

Protocols

- **Fire Monkey High Molecular Weight DNA Extraction from bacteria (no more than 1 billion cells)**

1. **Pellet the cells.** Remove all supernatant, after washing in 1x PBS (do not wash in TE as this could have a negative effect on yield).

2. **Lysozyme treatment.** Please follow the procedure below depending on whether you are using Gram positive or Gram negative bacteria:

    a. **For Gram positive bacteria** resuspend the pellet in 180µl of freshly prepared 20mg/ml Lysozyme solution (1.2% Triton X-100 in H_2O;) and incubate at 37^{0}C for 30 mins.

    b. **For Gram negative bacteria** incubate the pellet at 37^{0}C for 10 mins in 100µl of freshly prepared 3mg/ml Lysozyme solution (1.2% Triton X-100 in H_2O).

3. **For the extraction of:**

    a. **Total Nucleic Acid** go straight to step 4

    b. **DNA only** add 10µl of a 20mg/ml of RNase A solution (dilute in H_2O, no salts), mix by vortexing.

4. **Add 300µl of LSDNA and pipette thoroughly.**

5. **Add 20µl of 20mg/ml Proteinase K and vortex briefly.**

6. **Incubate at 56^{0}C for 20 mins. Once complete:**
    a. **For DNA-only** raise the temperature to 80^{0}C to pre-warm 2 x empty 1.5ml Eppendorfs per sample. Also, pre-warm another Eppendorf containing enough EB to perform 2 elutions per sample (~250µl per sample).
    b. **For Total Nucleic acid** (TNA) raise the temperature to 65^{0}C to pre-warm 2 x empty 1.5ml Eppendorfs per sample. Also, pre-warm another Eppendorf containing enough EB to perform 2 elutions per sample (~250µl per sample).

7. **Add 350µl of BS to the lysed sample and vortex briefly.** A white precipitate might form which will be dissolved in step 8.

8. **Add 400µl of 75% isopropanol and vortex briefly.** Make sure that the solution is clear before adding to the column.

9. **Use a wide bore tip to transfer 600µl of the sample to the spin column.**

10. **Centrifuge at 8,000rpm (4,722 X G) for 1 min.** Discard the flow-through.

11. **Use a wide bore tip to add the remainder of the sample to the spin column and centrifuge at 8,000rpm (4,722 X G) for 1 min.** Discard the flow-through.

12. **Add 500µl of WS to the spin column and centrifuge at 8,000rpm (4,722 X G) for 1 min.** Discard the flow-through.

13. **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.

14. **Centrifuge the spin column at 14,000rpm (14,462 X G) for 1 min.** Discard the flow-through.

15. **Transfer the column to a pre-warmed Eppendorf.**

16. a. **For DNA-only extraction add 100µl of EB (pre-warmed at 80^{0}C) to the spin column and incubate at 80^{0}C for 1 min. DO NOT** elute in a volume smaller than 100µl as this could have a negative effect on DNA integrity.

    b. **For TNA extraction add 100µl of EB (pre-warmed at 65^{0}C) to the spin column and incubate at 65^{0}C for 1 min. DO NOT** elute in a volume smaller than 100µl.

17. **Elute at 4,000rpm (1,180 X G) for 2 mins: Fraction A.** This fraction contains HMW DNA together with smaller DNA fragments and RNA. **DO NOT** re-load the eluate to re-elute in a more concentrated format as this could have a negative effect on DNA integrity.

18. **Transfer the column to a pre-warmed Eppendorf.**

19. **Add 80µl of fresh EB (for HMW pre-warmed to 80^{0}C/for TNA pre-warmed to 65^{0}C) to the spin column and incubate at 80^{0}C for DNA/65^{0}C for TNA, for 1 min. DO NOT** elute in a volume smaller than 80µl as this could have a negative effect on DNA integrity.

20. **Elute at 4,000rpm (1,180 X G) for 2 mins: Fraction B**. This fraction contains HMW-DNA with fewer smaller fragments than Fraction A.

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

- **Fire Monkey High Molecular Weight DNA Extraction from white blood cells and other mammalian cell types (no more than 5 million cells)**

Note: For tips on aliquoting blood prior to storage, or DNA extraction please refer to the troubleshooting section: Low DNA yield from white blood cells.

1. **Add 5x volumes of ice-cold Red Blood Cell lysis solution (150mM NH_4Cl, 10mM $NaHCO_3$, 0.1mM EDTA, pH 7.3) to up to 1ml of whole blood and mix thoroughly by vortexing.** For other mammalian cell types pellet the cells. Remove all supernatant, after washing in 1x PBS (do not wash in TE as this could have a negative effect on yield) and go straight to step 6.

2. **Incubate at room temperature for 5 to 10 mins and vortex frequently (3 to 5 times) during incubation.**

3. **Spin at 250 X G for 3 mins and discard supernatant without disturbing the pellet.**

4. **Add 2x of ice-cold Red Blood Cell lysis solution to the pellet and mix thoroughly by vortexing.**

5. **Spin at 250 X G for 3 mins and discard supernatant without disturbing the pellet.** Pellets can stay on ice for up to 1 hr prior to use.

6. **Add 300µl of LSDNA and pipette thoroughly.**

7. **Add 20µl of 20mg/ml Proteinase K and vortex briefly.** For the extraction of **DNA-only, also add 10µl of a 20mg/ml of RNase A solution** (dilute in H_2O, no salts as this could have a negative effect on extract yield and integrity) and vortex briefly.

8. **Incubate at 56°C for 10 mins. Once complete:**
    a. **For DNA-only** raise the temperature to 80°C to pre-warm 2 x empty 1.5ml Eppendorfs per sample. Also, pre-warm another Eppendorf containing enough EB to perform 2 elutions per sample (~250µl per sample)

    b. **For Total Nucleic Acid (TNA)** raise the temperature to 65°C to pre-warm 2 x empty 1.5ml Eppendorfs per sample. Also, pre-warm another Eppendorf containing enough EB to perform 2 elutions per sample (~250µl per sample)

9. **Add 350µl of BS and vortex briefly.** A white precipitate might form which will be dissolved on step 10.

10. **Add 400µl of 75% isopropanol and vortex briefly**. Make sure that the solution is clear before adding to the column.

11. **Use a wide bore tip to transfer 600µl of the sample to the spin column.**

12. **Centrifuge at 8,000rpm (4,722 X G) for 1 min.** Discard the flow-through.

13. **Use a wide bore tip to add the remainder of the sample to the spin column and centrifuge at 8,000rpm (4,722 X G) for 1 min. Discard the flow through.**

14 **Add 500µl of WS to the spin column and centrifuge at 8,000rpm for 1 min.** Discard the flow-through.

15. **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.

16. **Centrifuge the spin column at 14,000rpm (14,462 X G) for 1 min.** Discard the flow-through.

17. **Transfer the column to a pre-warmed Eppendorf.**

18. a. **For DNA-only extraction add 100µl of EB (pre-warmed at 80^{0}C) to the spin column and incubate at 80^{0}C for 1 min. DO NOT** elute in a volume smaller than 100µl as this could have a negative effect on DNA integrity.

    b. **For TNA extraction add 100µl of EB (pre-warmed at 65^{0}C) to the spin column and incubate at 65^{0}C for 1 min. DO NOT** elute in a volume smaller than 100µl.

19. **Elute at 4,000rpm (1,180 X G) for 2 mins: Fraction A.** This fraction contains HMW DNA together with smaller DNA fragments and RNA. **DO NOT** re-load the eluate to re-elute in a more concentrated format as this could have a negative effect on DNA integrity.

20. **Transfer the column to a pre-warmed Eppendorf.**

21. **Add 80µl of fresh EB (for HMW pre-warmed to 80^{0}C/for TNA pre-warmed to 65^{0}C) to the spin column and incubate at 80^{0}C for DNA-only/65^{0}C for TNA, for 1 min. DO NOT** elute in a volume smaller than 80µl as this could have a negative effect on DNA integrity.

22. **Elute at 4,000rpm (1,180 X G) for 2 mins: Fraction B.** This fraction contains HMW-DNA with fewer smaller fragments than Fraction A.

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

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. **Pre-warm 2x 1.5ml empty Eppendorfs to 80^{0}C per extracted DNA sample. Also, pre-warm another Eppendorf containing enough EB to perform 2 elutions per sample ~250µl/sample at 80^{0}C**.

3. **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.**

4. **Use wide bore tips to transfer 600µl to the spin column.**

5. **Centrifuge at 8,000rpm (4,722 X G) for 1min.** Discard the flow-through.

6. **Use wide bore tips to transfer the remainder of the sample to the spin column.**

7. **Centrifuge at 8,000rpm (4,722 X G) for 1min**. Discard the flow-through.

8. **Add 500µl of WS to the spin column and centrifuge at 8,000rpm (4,722 X G) for 1 min.** Discard the flow-through.

9. **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.

10. **Centrifuge the spin column at 14,000rpm (14,462 X G) for 1 min.** Discard the flow-through.

11. **Transfer the column to the pre-warmed Eppendorf.**

12. **Add 100µl of EB (pre-warmed at 80^{0}C) to the spin column and incubate at 80^{0}C for 1 min. DO NOT** elute in a volume smaller than 100µl as this could have a negative effect on DNA integrity.

13. **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.

14. **Transfer the column to the pre-warmed Eppendorf.**

15. **Add 80µl of EB (pre-warmed at 80^{0}C) to the spin column and incubate at 80^{0}C for 1 min. DO NOT** elute in a volume smaller than 80µl as this could have a negative effect on DNA integrity.

16. **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

Questions	Solutions
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 at 11,000rpm (8,928 X G) for 3mins unless otherwise stated.
How long should I vortex the sample?	The Fire Monkey protocol requires three vortexing steps. The duration of vortexing should be up to 5 seconds. If vortexing is omitted this will have a negative effect on DNA strand length and yield. We recommend that you avoid excessive vortexing (i.e. longer than 20 seconds) 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, increase the duration of vortexing (i.e. 20 seconds) and/or pipette and shake vigorously.
How can I achieve maximal DNA yield for hard to lyse samples?	For hard to lyse samples we recommend the 56°C lysis incubation should be performed on a shaking incubator with short vortexing steps every 5 to 10 minutes during lysis. Additionally, the lysis time can also be increased.

Questions	Solutions
What is the maximal starting material I can use for the Fire Monkey Protocol?	Overloading the column should be avoided. In general loading more $5x10^6$ mammalian cells, or 10^9 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 cell type.
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.
After loading, a small volume of lysed material has remained in the column, what should I do?	This means that the column was overloaded. Repeat the initial loading spin at 8,000rpm for 1 minute and if the problem persists perform successive spins at 11,000rpm for 1 minute until all lysed material has passed through the column.
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 this 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.

Questions	Solutions
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. Alternatively, DNA concentration can be increased by reducing the eluted volume by evaporation at 65°C (lid open, ~100 to ~50µl after ~30 mins of evaporation). 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, if the precipitate occurs, 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).

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