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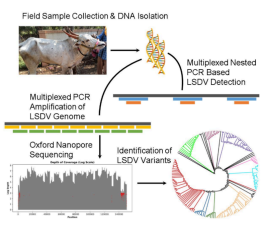
Protocol for Detection and Genotyping of Lumpy Skin Disease Virus (LSDV) using Oxford Nanopore Sequencing



Virus Evolution

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Dhanasekaran Shanmugam[1,2], Manali Bajpai[1,2], Ajinkya Khilari[1,2], Bhagyashree Likhitkar[1,2]

[1]Biochemical Sciences Division, CSIR- National Chemical Laboratory, Pune - 411008, India;
[2]Academy of Scientific and Innovative Research (AcSIR), Ghaziabad - 201002, India

Dhanasekaran Shanmugam: Corresponding author;

Dhanasekaran Shanmugam
CSIR-NCL, Pune

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We use this protocol, and it's working.

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Abstract

The protocol relates to nested multiplex PCR-based detection method for disease surveillance. In particular, the present disclosure provides a nested multiplex detection primer panel (NMDPP) to detect Lumpy Skin Disease Virus (LSDV) from the cattle sample. The present invention also provides a rapid, sensitive, and improved nested multiplex polymerase chain reaction employing said primer panel for detecting LSDV and its variants. The present disclosure relates to whole genome multiplex PCR-based genotyping method for viral variant studies. In particular, the present disclosure provides a multiplex primer panel (LSDV_WGSPP_3.5) to PCR amplify and genotype Lumpy Skin Disease Virus (LSDV) from the given cattle sample.

Guidelines

You must have read, understood, and follow the health and safety instructions before performing the protocol.

1. **Sample Collection**: Use sterile swabs and tubes to collect nasal and skin samples. Properly restrain animals during sample collection with assistance from trained personnel. Store collected samples in tubes containing 1X PBS and ensure they are properly submerged. Label sample tubes with cryo-resistant markers or barcoded stickers.
2. **Transportation and Storage**: Transport samples at 4°C using cooler boxes with ice packs. Store samples at -80°C until DNA isolation. Ensure samples are properly sealed to prevent spillage and cross-contamination.
3. **DNA Isolation**: Follow the manufacturer's protocol for DNA isolation instruments (e.g., MagNA Pure 96). Label samples accurately using the same tracking number for consistency. Store isolated DNA at -20°C until further use.
4. **DNA Quantification**: Use Qubit and Nanodrop methods for DNA quantification to ensure quality.
5. **PCR Setup**: Store primer pools and DNA samples at -20°C after use to maintain stability.
6. **Bead Purification**: Use freshly prepared 80% ethanol for washing beads. Mix beads by pipetting gently to avoid DNA shear. Keep the sample on a magnetic stand during washing and elution steps.
7. **Oxford Nanopore Sequencing**: Use at least 50 ng of high-quality DNA for sequencing. Follow Oxford Nanopore Technologies' instructions for flow cell priming and sample loading. To preserve sequencing quality, avoid introducing air bubbles during flow cell priming and sample loading. Ensure all software and tools are updated before starting the analysis.

Following these guidelines, you can ensure a reliable and reproducible protocol for detecting and genotyping LSDV using Oxford Nanopore Sequencing.

Materials

1. GeneRuler 100 bp Plus DNA Ladder, ready-to-useThermo Fisher ScientificCatalog #SM0323
2. Rapid Barcoding Kit 96 V14 (SQK- RBK114.96)Oxford Nanopore TechnologiesCatalog #SQK- RBK114.96
3. Qubit FluorometerInvitrogen - Thermo FisherCatalog #Q32866
4. AMPure XPBechman CoulterCatalog #A63882
5. RepliQa HiFi ToughMix® VWR InternationalCatalog #95200-500
6. EmeraldAmp® GT PCR Master MixTakara Bio Inc.Catalog #RR310A
7. 1X TAE Buffer
8. Invitrogen™ UltraPure™ AgaroseInvitrogen - Thermo FisherCatalog #16500100
9. Ethidium bromide 10 mg/mlMerck MilliporeSigma (Sigma-Aldrich)Catalog #E1510
10. MagNA Pure 96 DNA and Viral NA LV KitRocheCatalog #06 374 891 001
11. Qubit™ dsDNA HS Assay KitInvitrogen - Thermo FisherCatalog #Q32851
12. Qubit™ Assay TubesInvitrogen - Thermo FisherCatalog #Q32856

Protocol materials

- 1X TAE Buffer
- Invitrogen™ UltraPure™ Agarose **Invitrogen - Thermo Fisher** **Catalog #**16500100
- GeneRuler 100 bp Plus DNA Ladder, ready-to-use **Thermo Fisher Scientific** **Catalog #**SM0323
- Ethidium bromide 10 mg/ml **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**E1510
- Rapid Barcoding Kit 96 V14 (SQK- RBK114.96) **Oxford Nanopore Technologies** **Catalog #**SQK- RBK114.96
- MagNA Pure 96 DNA and Viral NA LV Kit **Roche** **Catalog #**06 374 891 001
- Qubit™ dsDNA HS Assay Kit **Invitrogen - Thermo Fisher** **Catalog #**Q32851
- Qubit Fluorometer **Invitrogen - Thermo Fisher** **Catalog #**Q32866
- Qubit™ Assay Tubes **Invitrogen - Thermo Fisher** **Catalog #**Q32856
- AMPure XP **Bechman Coulter** **Catalog #**A63882
- RepliQa HiFi ToughMix® **VWR International (Avantor)** **Catalog #**95200-500
- EmeraldAmp® GT PCR Master Mix **Takara Bio Inc.** **Catalog #**RR310A

Safety warnings

This protocol does not require any hazardous substances or infectious agents. However, it includes DNA extracted from cattle skin swab and nasal swab samples.

You must always wear safety equipment, including lab coats, gloves, and safety goggles, when handling chemicals and biological agents. While the extracted DNA does not include any infectious agents, treat them carefully. Do not remove them from the laboratory. Use a dedicated notebook and pen to make notes during the experiments. Do not put anything into your mouth while in the laboratory. Wash your hands each time you leave the laboratory.

1. **Biohazard Risk**: Handling samples from infected animals can pose biohazard risks. Use appropriate personal protective equipment (PPE) such as gloves, lab coats, and masks to prevent exposure.
2. **Cross-Contamination**: Always change gloves when handling each sample to avoid cross-contamination. Work aseptically to prevent contamination between samples during collection, processing, and PCR setup.
3. **Sample Degradation**: Improper storage or temperature fluctuations during transportation can lead to sample degradation, affecting DNA quality. Maintain a consistent temperature of 4°C during transport and store samples at -80°C until further processing.
4. **DNA Integrity**: Poor quality or degraded DNA (e.g., $A_{260/280}$ ratio below 1.8) can lead to inaccurate PCR results. Verify DNA integrity before proceeding to PCR or sequencing.
5. **Primer Handling**: Avoid cross-mixing between outer and inner primer pools, which can lead to non-specific amplification. Thaw primer pools on ice and gently vortex them before use to ensure even mixing.
6. **Bead Purification**: Avoid over-drying beads during purification, as this can result in loss of DNA. Handle beads gently with pipetting to avoid DNA shearing, which can compromise sequencing quality.
7. **Flow Cell Handling**: Introducing air bubbles during flow cell priming and loading can result in pore loss, reducing sequencing efficiency. Follow pipetting instructions carefully and avoid using excessive force to prevent damage to the flow cell.
8. **Chemical Handling**: Handle ethidium bromide and other staining agents used in gel electrophoresis cautiously, as they are toxic and mutagenic. Dispose of all hazardous materials following appropriate safety protocols.
9. **Instrument Usage**: Follow the manufacturer's guidelines strictly for each device.

Adhering to these warnings will help ensure safety and accuracy during the detection and genotyping of LSDV.

Ethics statement

This protocol for the detection and genotyping of Lumpy Skin Disease Virus (LSDV) involves the collection of biological samples from cattle, which requires adherence to ethical guidelines. Prior to commencing this protocol, it is essential to obtain approval from the user's Institutional Animal Care and Use Committee (IACUC) or an equivalent ethics committee(s). This approval ensures that all animal handling, sample collection, and transportation procedures are conducted to minimise stress and discomfort to the animals.
The protocol must be carried out by the principles outlined in the IACUC-approved guidelines, including proper restraint techniques, the use of trained personnel for sample collection, and maintaining sterile conditions to ensure the well-being of the animals. This commitment to ethical standards ensures the research's integrity and the animals' welfare.

Before start

1. **Personal Protective Equipment (PPE)**: Gather lab coats, gloves, masks, and eye protection for all personnel handling samples to ensure biosafety and prevent contamination.
2. **Sample Collection Materials**: Prepare sterile flocked swabs and 15 mL collection tubes containing 400 µL of 1X PBS. Label tubes using cryo-resistant permanent markers or cryo-compatible barcoded stickers. Arrange cooler boxes with ice packs for transporting samples at 4°C.
3. **DNA Isolation Equipment and Reagents**: Set up the DNA isolation instrument (e.g., MagNA Pure 96) according to the manufacturer's protocol. Ensure the DNA and Viral NA LV Kit (Roche) or other chosen kits for DNA extraction are available.
4. **PCR Setup Materials**: Prepare and label outer and inner primer pools for nested PCR. Thaw and mix the primers on ice before use. Stock up on EmeraldAmp® GT PCR Master Mix and nuclease-free water for PCR reactions. Calibrate thermal cyclers and ensure they are ready for use.
5. **Reagents for DNA Quantification**: Prepare Qubit™ dsDNA HS Assay Kit, Qubit Fluorometer, and Nanodrop instrument. Calibrate the Qubit Fluorometer and Nanodrop for accurate readings. Ensure all reagents for Qubit and Nanodrop assays are thawed and ready.
6. **Gel Electrophoresis Setup**: Prepare 1% agarose gel, 1X TAE buffer, ethidium bromide or other DNA stains, and a DNA ladder.
7. **Bead Purification Materials**: Prepare AMPure XP beads and freshly prepared 80% ethanol for DNA purification. Set up a magnetic stand for bead separation and have pipettes ready to handle beads.
8. **Sequencing Kit and Flow Cells**: Ensure the availability of the Oxford Nanopore SQK-RBK114.96 kit and flow cells (version 10). Dilute rapid adapters according to the kit instructions. Verify that the MinKNOW software is installed and up to date on the sequencing computer.
9. **General Laboratory Setup**: Clean and disinfect all work surfaces to minimize contamination risk. Arrange all reagents, pipettes, tubes, and equipment in a sterile environment. Verify that all instruments, including the centrifuge, pipettes, and vortex mixers, are calibrated and functioning properly.

Having all these preparations in place ensures a smooth workflow and minimizes the risk of errors during the protocol execution for LSDV detection and genotyping.

1. Sample Collection and Storage

10m

1 **For nasal swab samples**

5m

This method can be used for sample collection from symptomatic as well as asymptomatic cattle.

- Insert flocked swab (HiMedia, India) inside the nostril of the cattle to take the mucus sample
- Remove the swab carefully and insert the swab inside 15 ml sample collection tubes containing
  400 µL of 1X PBS
- Make sure that the swab head containing the sample is fully submerged in 1X PBS
- The tube can be marked either with cryo-resistant permanent marker or preferably using cryo-compatible barcoded sticker

Precautions

- Use protective clothing and work aseptically avoiding cross-contamination between samples
- Change gloves after each sample collection
- Nasal swabs must be collected using sterile swabs and placed into sterile tubes containing 1X PBS for transportation

 # NOTE: Animal has to be properly restrained with help from trained personnel for nasal swab collection

2 **For skin scab samples**

5m

This method can be used for sample collection from symptomatic cattle which have open wounds on skin nodules.

- Find the lesion on the cattle and clean the area identified for sample collection by gently spraying sterile water
- Place a swab on the wound and rub gently to remove the top layer including any dirt that may be on the wound
- Take fresh swab and rub to collect the oozing fluid from the lesion
- Place the swab inside 15 ml sample collection tubes containing
  400 µL of 1X PBS
- Make sure that the swab head containing the sample is fully submerged in 1X PBS
- The tube can be marked either with cryo-resistant permanent marker or preferably using cryo-compatible barcoded sticker

Precautions

- Use protective clothing and work aseptically avoiding cross-contamination between samples

- Change gloves after each sample collection
- Collected skin swab samples must be placed into sterile tubes containing 1X PBS for transportation

3 **Transportation and storage**

The samples must be transported to the laboratory in cooler boxes maintaining 4 °C using ice packs.

Store the samples in -80 °C until the time it is processed for DNA isolation.

Precautions

- Samples must be properly sealed to avoid spillage and cross contamination
- It is must to maintain the temperature at 4 °C while transportation to avoid sample degradation
- Make sure that sample containing tubes are properly marked in a permanent manner

2. DNA Isolation, Quantification and Storage

3h

4 **DNA isolation**

3h

- **# NOTE:** Multiple protocols and kits are available for viral DNA isolation from samples obtained using swabs. The method and protocol given here have been optimised using the Roche MagNA Pure 96 Instrument and compatible kits. Other DNA isolation instruments and methods can be used as per users' choice.

- Isolate DNA using MagNA Pure 96 DNA and Viral NA LV Kit **Roche Catalog #**06 374 891 001 as per manufacturer's protocol of MagNA Pure 96 Instrument

Equipment	
MagNA Pure 96 Instrument	NAME
Automated nucleic acid purification	TYPE
Roche Molecular Systems, Inc	BRAND
06541089001	SKU

- Take proper precautions as per instrument manual while DNA isolation
- Label the samples properly using the same tracking number/barcode as previously used on the sample collection vials. This is critical for sample tracking and linking to metadata

5 **Qualitative and Quantitative Analysis of DNA** 10m

- DNA quantification must be carried out using Qubit method as given in 5.1
- Purity and integrity of DNA samples can be determined using a Nanodrop instrument as given in 5.2

5.1 Reagents used for DNA quantification using Qubit method - 5m

- Qubit™ dsDNA HS Assay Kit **Invitrogen - Thermo Fisher Catalog #**Q32851
- Qubit Fluorometer **Invitrogen - Thermo Fisher Catalog #**Q32866
- Qubit™ Assay Tubes **Invitrogen - Thermo Fisher Catalog #**Q32856

DNA quantification steps -

 # NOTE: Select the option for DNA quantification in the fluorometer

- Take 190 µL of HS buffer and 10 µL of standards 1 and 2 in separate assay tubes labelled as S1 and S2 respectively
- For each sample, take 199 µL of HS buffer in assay tube and add 1 µL of the sample to be quantified
- The assay tubes must be vortexed to mix the contents and readings are taken using the fluorometer

- **# NOTE:** DNA concentration of 10ng/µL or above is desirable for further steps

5.2 DNA quantification using Nanodrop instrument - 5m

 # NOTE: Select the option for DNA quantification in the Nanodrop instrument

- Set the blank twice with 1 µL of blank reagent
- Take the readings using 1 µL of each sample
- Reading must be taken in ng/µL
- $A_{260/280}$ must be 1.8 and $A_{260/230}$ must be between 2 to 2.2 for good quality DNA samples

 # NOTE: For samples which do not have the required quality and quantity of DNA the extraction can be repeated

6 **Storage**

- After isolation and quantification store the samples in -20 °C until further use

24w

3. Nested PCR using Multiplex Diagnostic Primer Panel (LSDV_NMDPP)

6h 30m

7 For the detection of the Lumpy Skin Disease Virus (LSDV), the following nested multiplex PCR protocol is used.

- Three distinct LSDV genomic loci are targeted by nested PCR. The PCRs are multiplexed in a single reaction.
- The primers for the nested PCRs are designed using the reference genome NC_003027.1 for Lumpy skin disease virus NI-2490 available in NCBI
- The PCR mix EmeraldAmp® GT PCR Master Mix **Takara Bio Inc. Catalog #**RR310A is recommended for the nested PCRs
- Details of nested PCR primer sequences are mentioned below in **Table 01**

	A	B	C	D	E	F	G	H	I
								Genomic Position	
	S. No.	**Name**	**Pool**	**Sequence**	**Length**	**Tm (°C)**	**% GC content**	**Start**	**End**
	1	LSDV_NMDPP_1	Outer	TACATACATTTCAAGTACTAAAGAGAAGGAA	31	56.2	29	14452	14482
	2	LSDV_NMDPP_2	Outer	ATGTCAACAACATTTTTGCTATTCAATG	28	55.9	29	13516	13543
	3	LSDV_NMDPP_3	Outer	AACGAGTTGTTAGTCATTTGAGATAC	26	55.0	35	80177	80202
	4	LSDV_NMDPP_4	Outer	ACCATTCCAGAATTAAGTTTGATAATAAAC	30	54.8	27	79082	79111
	5	LSDV_NMDPP_5	Outer	TAAACATAGACTCTTCTTTCGGTAGAC	27	55.8	37	132962	132988

	A	B	C	D	E	F	G	H	I
	6	LSDV_NMDPP_6	Outer	GTTCGGTTTCATATTTTTAGCATATTCAC	29	55.5	31	131714	131742
	7	LSDV_NMDPP_7	Inner	CAACATCTAACGAAAAAACATTATCGTC	28	55.3	32	13863	13890
	8	LSDV_NMDPP_8	Inner	AATATTCTTTTCCGGATCATTCGC	24	55.5	38	14303	14326
	9	LSDV_NMDPP_9	Inner	AAGAACATAATCTTGTATCATCGCC	25	55.0	36	79310	79334
	10	LSDV_NMDPP_10	Inner	ACTGTGACCATTGATCCGTTATC	23	56.3	43	79756	79778
	11	LSDV_NMDPP_11	Inner	GGCTATACGCATAGTGATATTAAAGC	26	55.3	38	132069	132094
	12	LSDV_NMDPP_12	Inner	CATAATCTGGAATAGAATCGTATTGTAATG	30	54.2	30	132475	132504

Table 01 - Details of LSDV_NMDPP primer set

- For individual primers, 100µmolar stocks are prepared in Nuclease Free Water and stored in -20 °C
- For multiplexed nested PCR reaction, the outer and inner primer pools are prepared by separately mixing each of the outer primers in one pool and each of the inner primers in the other pool. The primer pools can be stored in -20 °C
- At the time of setting up the PCR reactions, the outer and inner primer pools are diluted 10 times (10µmolar primer mix) with Nuclease Free Water and added to the reactions as given in **Table 02** and **Table 03**

 # NOTE: Primer pools can be ordered directly from the manufacturer as ready-to-use 10µmolar primer mix.

Precautions

- Make sure to avoid cross-mixing of primers between the outer and inner primer pools
- Primer mix and DNA samples must be thawed on ice
- Gently mix primer pools (vortexing) and DNA samples (flicking) and spin down before use

- After every use, store primer mix and DNA samples in -20 °C to prevent degradation

8 **Nested PCR setup using LSDV_NMDPP outer primer mix** 3h

 # NOTE: For nested PCR, the first PCR reaction is carried out using outer primer pool and the second PCR reaction is carried out using inner primer pool

- The composition of the PCR reaction mix using outer primer pool is given in **Table 02**

	A	B
	EmeraldAmp® GT PCR Master Mix	12.5 µL
	LSDV_NMDPP outer primer mix	3.75 µL
	Template DNA	50ng
	Nuclease Free Water	Makeup volume to 25 µL

Table 02 – PCR reaction mix composition using LSDV_NMDPP outer primers

- PCR amplification conditions for outer primer pool reaction is given in **Table 03**

	A	B	C	D
	Step	**Temperature**	**Time**	**Cycles**
	Initial Denaturation	94 °C	5 min	X 1 cycle
	Denaturation	94 °C	30 sec	X 25 cycles
	Annealing	50 °C	30 sec	
	Extension	72 °C	2 min	
	Final Extension	72 °C	4 min	X 1 cycle
	Hold	4 °C	∞	

Table 03 – PCR conditions for reaction using LSDV_NMDPP outer primers

- The product of the first PCR using outer primer pool will amplify ~1kb region from LSDV genome

9 **Nested PCR setup using LSDV_NMDPP inner primers** 2h 30m

- The composition of the PCR reaction mix using inner primer pool is given in **Table 04**

	A	B
	EmeraldAmp® GT PCR Master Mix	12.5 µL
	LSDV_NMDPP inner primer mix	3.75 µL
	Template DNA	5 µL of outer primer PCR product
	Nuclease Free Water	Makeup volume to 25 µL

Table 04 – PCR reaction mix composition using LSDV_NMDPP inner primers

- PCR amplification conditions for inner primer pool reaction is given in **Table 05**

	A	B	C	D
	Step	**Temperature**	**Time**	**Cycles**
	Initial Denaturation	94 °C	5 min	X 1 cycle
	Denaturation	94 °C	30 sec	X 30 cycles
	Annealing	55 °C	30 sec	
	Extension	72 °C	1 min 15 sec	
	Final Extension	72 °C	3 min	X 1 cycle
	Hold	4 °C	∞	

Table 05 – PCR conditions for reaction using LSDV_NMDPP inner primers

- The product of the second PCR using inner primer pool will amplify ~0.5kb region from the target region of LSDV genome

10 **Analysis of PCR products by agarose gel electrophoresis**

30m

The second PCR product is analyzed using 1% agarose gel as followed -

- Reagents required are -

Invitrogen™ UltraPure™ Agarose **Invitrogen - Thermo Fisher Catalog #**16500100

GeneRuler 100 bp Plus DNA Ladder, ready-to-use **Thermo Fisher Scientific Catalog #**SM0323

Ethidium bromide 10 mg/ml **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**E1510

1X TAE Buffer

- 5 µL of PCR product is loaded on the agarose gel and electrophoresis is carried out at 120mV for 00:30:00
- Samples are considered positive for LSDV presence if 0.5kb band is detected
- Positive samples are processed for whole genome amplification of LSDV using LSDV_WGSPP_3.5 primer panel

4. Multiplexed PCR Amplification using **LSDV W**hole **G**enome **S**equencing Primer **P**anel **3.5 (LSDV_WGSPP_3.5)**

5h

11 PCR amplification of whole genome of LSDV from field samples is carried out for genotyping. The PCR amplification of overlapping ~3.5kb fragments is done in multiplexed manner.

4h

- The PCR primers for the whole genome amplication of LSDV were designed using the reference genome **NC_003027.1** for Lumpy skin disease virus NI-2490 available in NCBI using **PrimalScheme**
- A total of 48 primer pairs were designed to cover the entire LSDV genome and each PCR product is ~3.5kb in size with ~0.5kb overlap between adjacent PCR fragments
- The **LSDV_WGSPP_3.5** primer set is divided into pool A and pool B such that adjacent amplicons are in different pools. The details of the entire primer set is given in **Table 06**

	A	B	C	D	E	F	G	H	I
								Genomic Position	
	S. No.	**Primer Name**	**Pool**	**Primer Sequence**	**Size**	**% GC Content**	**Tm**	**Start**	**End**
	1	LSDV_WGSPP_1_F	A	AGTGCTGATTTCACTAGCGAAATATC	26	38.46	60.23	286	311
	2	LSDV_WGSPP_1_R	A	ACGTAGTTCGATGCTATTTTTCCATTT	27	33.33	60.64	3688	3714
	3	LSDV_WGSPP_2_F	B	GTTGTACCCTTGACGCGTTTTC	22	50	60.78	3370	3391

	A	B	C	D	E	F	G	H	I
	4	LSDV_WGSPP_2_R	B	TGGAAGGGAGTGATAATACCAACAC	25	44	60.73	6904	6928
	5	LSDV_WGSPP_3_F	A	GAAGGGAATATGCAATCGCGGA	22	50	61.31	6551	6572
	6	LSDV_WGSPP_3_R	A	CAACTATGGTTTGGAAGCACTTGT	24	41.67	60.28	10123	10146
	7	LSDV_WGSPP_4_F	B	CACGGATTGTAGACAACCAAAGC	23	47.83	60.61	9829	9851
	8	LSDV_WGSPP_4_R	B	ACGATGTGTACGATGTTGCAGA	22	45.45	60.53	13369	13390
	9	LSDV_WGSPP_5_F	A	TCGTAAGTATTTATCCACACCACCT	25	40	59.96	12732	12756
	10	LSDV_WGSPP_5_R	A	GGCCGCCGTAACTAATGTGATT	22	50	61.5	16323	16344
	11	LSDV_WGSPP_6_F	B	TCGTCTTTAGATGGGGTAGAGTTACT	26	42.31	61.03	15858	15883
	12	LSDV_WGSPP_6_R	B	TTTTTACGCCATTGACCTCAATAGAT	26	34.62	59.84	19340	19365
	13	LSDV_WGSPP_7_F	A	AACCCCATTAAGAAAGCCACTCA	23	43.48	60.44	18471	18493
	14	LSDV_WGSPP_7_R	A	TGGAGCAGGATGTAGACTCGTT	22	50	61.07	21932	21953
	15	LSDV_WGSPP_8_F	B	TCCACCAGTTAAAGAGGCGTTG	22	50	60.99	21566	21587
	16	LSDV_WGSPP_8_R	B	CCGAGTTTCACATGTTGAAGTCAC	24	45.83	60.86	25092	25115
	17	LSDV_WGSPP_9_F	A	CGTCATCTGTGCAACGTTCATG	22	50	60.9	24748	24769
	18	LSDV_WGSPP_9_R	A	TGTGATCTTATACGTACGACAAATGGA	27	37.04	60.9	28265	28291
	19	LSDV_WGSPP_10_F	B	CACAAGTTCATACGAATCTACTTCATCAC	29	37.93	61.25	27887	27915
	20	LSDV_WGSPP_10_R	B	CGTCCTCTACACATTTCAAATGCG	24	45.83	61.03	31263	31286
	21	LSDV_WGSPP_11_F	A	TCCTGAGTACTTTGACCCAGACA	23	47.83	60.95	30859	30881

	A	B	C	D	E	F	G	H	I
	22	LSDV_WGSPP_11_R	A	CTAGTCATAAAGGCGTTGGTGGT	23	47.83	60.87	34408	34430
	23	LSDV_WGSPP_12_F	B	TCCCAGTAGATAAAACTTCAGAAAACGA	28	35.71	61.23	34167	34194
	24	LSDV_WGSPP_12_R	B	ACCCAATGTTAGTATCCAACGCA	23	43.48	60.56	37645	37667
	25	LSDV_WGSPP_13_F	A	TGAGCATCCTAGGGAAATTGTCG	23	47.83	60.69	37341	37363
	26	LSDV_WGSPP_13_R	A	TCAGTTATTCAGACGAACATAACGACT	27	37.04	61.06	40845	40871
	27	LSDV_WGSPP_14_F	B	AGGCACATCAAACGTCGATTCT	22	45.45	60.79	40602	40623
	28	LSDV_WGSPP_14_R	B	TATCGTCCTCTAGTGTCGCTGT	22	50	60.6	43968	43989
	29	LSDV_WGSPP_15_F	A	CACTAAACGAATCACCAATTTCACTGA	27	37.04	61.01	43735	43761
	30	LSDV_WGSPP_15_R	A	GGTGGAATAATATCAAATCCCTAAATGGC	29	37.93	61.38	47126	47154
	31	LSDV_WGSPP_16_F	B	CTGTTACTGTTCCAGACAAAGAATCG	26	42.31	60.78	46887	46912
	32	LSDV_WGSPP_16_R	B	TCGAGCCCGATAACAGAAAACG	22	50	61.16	50382	50403
	33	LSDV_WGSPP_17_F	A	TTTTAGGCGTTACTCTTCCCACTT	24	41.67	60.53	50050	50073
	34	LSDV_WGSPP_17_R	A	CTCGCCTTTGTGGTTACATCCA	22	50	61.05	53643	53664
	35	LSDV_WGSPP_18_F	B	GAACAAACTGCTGAAGCCACTG	22	50	60.72	53219	53240
	36	LSDV_WGSPP_18_R	B	GGGTCTATATATGTTATCGGCTCTGG	26	46.15	60.68	56692	56717
	37	LSDV_WGSPP_19_F	A	TGATGAAACAGCTATGTCTGATATCGA	27	37.04	60.75	56403	56429
	38	LSDV_WGSPP_19_R	A	GCCATGTAAAAGATCAGATGCGC	23	47.83	61.1	59783	59805

	A	B	C	D	E	F	G	H	I
	39	LSDV_WGSPP_20_F	B	CACTGGTATTGAAATATCTGATAGAGCAG	29	37.93	60.52	59481	59509
	40	LSDV_WGSPP_20_R	B	CTGGATTCAATTCTCCTAAACATACAAACT	30	33.33	60.98	63002	63031
	41	LSDV_WGSPP_21_F	A	GGCGGGACCCCTATAGGTATTAT	23	52.17	60.89	62779	62801
	42	LSDV_WGSPP_21_R	A	TTCCGTTACCACTTGCTTCCAG	22	50	60.99	66143	66164
	43	LSDV_WGSPP_22_F	B	GTGTTTCGCAATAGAACTTTTTGCC	25	40	60.7	65656	65680
	44	LSDV_WGSPP_22_R	B	CTTGAGATTTGCCACATTCCGC	22	50	60.91	69137	69158
	45	LSDV_WGSPP_23_F	A	CGAAGATCACGATGACGAAAACG	23	47.83	60.78	68758	68780
	46	LSDV_WGSPP_23_R	A	AAAATTCCCAACGGGCCTCTAG	22	50	60.8	72357	72378
	47	LSDV_WGSPP_24_F	B	ACGGGGATTTCCTAAAGAGCATG	23	47.83	60.94	71977	71999
	48	LSDV_WGSPP_24_R	B	TCGCGTCCCAACTATTCTTAGTG	23	47.83	60.68	75609	75631
	49	LSDV_WGSPP_25_F	A	TCTTCTCGGGAATGTGGTACGA	22	50	61.06	75374	75395
	50	LSDV_WGSPP_25_R	A	ACTGAGAAAAGGTATCCGGAATTGT	25	40	60.67	78745	78769
	51	LSDV_WGSPP_26_F	B	CAGAATATAATGGGTCACAAGGGACA	26	42.31	61.08	78524	78549
	52	LSDV_WGSPP_26_R	B	CTCGTGTGCAGATGGTATCCAA	22	50	60.6	81934	81955
	53	LSDV_WGSPP_27_F	A	TCATCATCAACTGTAGATTCGCCA	24	41.67	60.46	81673	81696
	54	LSDV_WGSPP_27_R	A	CGAACTCAGCTTTATCGGGTACA	23	47.83	60.68	85138	85160
	55	LSDV_WGSPP_28_F	B	CGTTTGGAGGCACCTCGATAAA	22	50	61.11	84835	84856
	56	LSDV_WGSPP_28_R	B	ACTTACTGAGGAAGCACCAATTGT	24	41.67	60.77	88322	88345

	A	B	C	D	E	F	G	H	I
	57	LSDV_WGSPP_29_F	A	CCAGGTTTAGGAATCGAAACAAGTG	25	44	60.71	88070	88094
	58	LSDV_WGSPP_29_R	A	CGAATAACGGTAAGTCGATGACAAATT	27	37.04	60.94	91524	91550
	59	LSDV_WGSPP_30_F	B	ACTACAGCGTCAGATTTTAAAAACCAA	27	33.33	60.69	91245	91271
	60	LSDV_WGSPP_30_R	B	GCGCAGTTGATATCTTTAGGCATG	24	45.83	60.92	94768	94791
	61	LSDV_WGSPP_31_F	A	GCAAATGAGACAAAATTTCCATCTGTC	27	37.04	60.63	94455	94481
	62	LSDV_WGSPP_31_R	A	TCGCATGTGCTCCTCTATAGGT	22	50	60.94	97950	97971
	63	LSDV_WGSPP_32_F	B	GGCAAAAACGAAATCGCCATCA	22	45.45	61.1	97725	97746
	64	LSDV_WGSPP_32_R	B	GTGGCAACTTTGTCTCTGACGA	22	50	61.24	101090	101111
	65	LSDV_WGSPP_33_F	A	GGTCTTACAACCCTAATAAACTCAGAAC	28	39.29	60.45	100652	100679
	66	LSDV_WGSPP_33_R	A	TCCAGATCCGAGTTCCATCATAGT	24	45.83	61.02	104207	104230
	67	LSDV_WGSPP_34_F	B	GGCTTATCATGACTACAGAAAGCGA	25	44	61.34	103923	103947
	68	LSDV_WGSPP_34_R	B	AGTCGGTAGTATATGTGCCGGT	22	50	60.93	107474	107495
	69	LSDV_WGSPP_35_F	A	ACGCCACCAGAGTATTCACCTA	22	50	61.07	107112	107133
	70	LSDV_WGSPP_35_R	A	AGTGGCGAATACGGGCATTATG	22	50	61.31	110742	110763
	71	LSDV_WGSPP_36_F	B	ATGAATGCAAGGGGTGTGAAGG	22	50	61.33	110280	110301
	72	LSDV_WGSPP_36_R	B	AACTCAGATTCAGTATCGTCGTCAC	25	44	61.05	113730	113754
	73	LSDV_WGSPP_37_F	A	CAGCATTCGCAGGTTCCACTAT	22	50	61.18	113502	113523

	A	B	C	D	E	F	G	H	I
	74	LSDV_WGSPP_37_R	A	GCTTTATTAAAAATAACCATTACCCCACC	29	34.48	60.37	116973	117001
	75	LSDV_WGSPP_38_F	B	TGCACAAAAATAATTCCGAGTCAAACT	27	33.33	61.17	116643	116669
	76	LSDV_WGSPP_38_R	B	AAGGTGGACAACAACACTTTTTCC	24	41.67	60.64	120229	120252
	77	LSDV_WGSPP_39_F	A	AACTGAACTTGTTACATTGTGTGATGT	27	33.33	60.64	120007	120033
	78	LSDV_WGSPP_39_R	A	CGCAATTCCCTCCATCGTCAAT	22	50	61.5	123403	123424
	79	LSDV_WGSPP_40_F	B	CACCACGACTGAACCATTCACT	22	50	60.99	123066	123087
	80	LSDV_WGSPP_40_R	B	TCGGATAGAGTACTAGCATCTACAGAA	27	40.74	60.75	126666	126692
	81	LSDV_WGSPP_41_F	A	ATGAACACAAGAAGGACGTCGG	22	50	61.05	126259	126280
	82	LSDV_WGSPP_41_R	A	GCCCCCAGCAATCCTAATAACA	22	50	60.61	129784	129805
	83	LSDV_WGSPP_42_F	B	GAAGTAGGATCACGTTTTAAAGGCG	25	44	60.82	129346	129370
	84	LSDV_WGSPP_42_R	B	ACCTCTTTAGTTTCTTGTCCTTTCCA	26	38.46	60.8	132781	132806
	85	LSDV_WGSPP_43_F	A	GACTGACTCGCTAGGATCTAATTATCC	27	44.44	60.9	132413	132439
	86	LSDV_WGSPP_43_R	A	ACACGCATCGAGACAATTATTAAAATCT	28	32.14	60.75	135883	135910
	87	LSDV_WGSPP_44_F	B	CGAATTACGTAAATACTGCGATGTATCA	28	35.71	60.69	135547	135574
	88	LSDV_WGSPP_44_R	B	CTCTAATATCCCTCAACGACATACAGTT	28	39.29	61.02	139064	139091
	89	LSDV_WGSPP_45_F	A	GTTCAAAAGTGTCGTCAATGAATGTC	26	38.46	60.28	138760	138785

	A	B	C	D	E	F	G	H	I
	90	LSDV_WGSPP_45_R	A	TGTGTTTAAATCAGCGCCATTATCTAC	27	37.04	60.95	142308	142334
	91	LSDV_WGSPP_46_F	B	CATAAGACACCATGGAATACATTAGCG	27	40.74	60.84	142090	142116
	92	LSDV_WGSPP_46_R	B	ACAACACTATTTTCCGATGCAACG	24	41.67	60.93	145566	145589
	93	LSDV_WGSPP_47_F	A	TGGATTCTTGGCAATTTTATCAGAAGAA	28	32.14	60.61	144858	144885
	94	LSDV_WGSPP_47_R	A	GGATTCATTAACCCTATCATTGCCC	25	44	60.2	148375	148399
	95	LSDV_WGSPP_48_F	B	GGTGCCGAAACAACATTGCTATC	23	47.83	61.22	147157	147179
	96	LSDV_WGSPP_48_R	B	GTGGAAGCCAATTAAGTAGAAGCC	24	45.83	60.22	150708	150731

Table 06 - Details of LSDV_WGSPP_3.5 primer set

- For individual primers 100µmolar stocks are prepared in Nuclease Free Water and stored in -20 °C
- For multiplexed genomic PCR reaction the pool A and pool B are prepared by separately mixing 10 µL of each of the pool A primers in one pool and 10 µL of each of the pool B primers in the other pool. The primer pools can be stored in -20 °C
- At the time of setting up the PCR reactions the pool A and pool B primer pools are diluted 10 times (10µmolar primer mix) with Nuclease Free Water and added to the reactions as given in **Table 07**

 # NOTE: Primer pools can be ordered directly from the manufacturer as ready-to-use 10µmolar primer mix.

- Pool A and Pool B PCR reactions are set up separately using RepliQa HiFi ToughMix® **VWR International (Avantor) Catalog #**95200-500 master mix

	A	B
	repliQa HiFi ToughMix®	12.5 µL
	Primer Mix of Pool A or Pool B	3.75 µL
	Template DNA	50 ng
	Nuclease Free Water	Makeup volume to 25 µL

Table 07 - PCR reaction mix composition using LSDV_WGSPP_3.5

- PCR amplification conditions for genomic PCR reaction is given in **Table 08**

	A	B	C	D
	Step	**Temperature**	**Time**	**Cycles**
	Initial Denaturation	98 °C	30 sec	X 1 cycle
	Denaturation	98 °C	15 sec	X 35 cycles
	Annealing and Extension	65 °C	6 min	
	Hold	4 °C	∞	

Table 08 – PCR conditions for multiplex whole genome amplification using LSDV_WGSPP_3.5 panel

Precautions

- Make sure to avoid cross-mixing of primers between the pool A and pool B sets
- Primer mix and DNA samples must be thawed on ice
- Gently mix primer pools (vortexing) and DNA samples (flicking) and spin down before use
- After every use store primer mix and DNA samples in -20 °C to prevent degradation

12 **Analysis of PCR products by agarose gel electrophoresis**

30m

 # NOTE: This is an optional step and the PCR products can be directly sequenced without analysis by agarose gel electrophoresis. However, this step is recommended for two reasons- 1) to confirm that the pool A and pool B PCRs have worked for each sample before sequencing; 2) to ensure that the PCR product is devoid of primer dimers (this can affect sequence data quality).

- Reagents required are -

Invitrogen™ UltraPure™ Agarose **Invitrogen - Thermo Fisher Catalog #**16500100

GeneRuler 100 bp Plus DNA Ladder, ready-to-use **Thermo Fisher Scientific Catalog #**SM0323

Ethidium bromide 10 mg/ml **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**E1510

1X TAE Buffer

- 5 µL of PCR product is loaded on the agarose gel and electrophoresis is carried out at 120mV for 00:30:00
- A 3.5kb band should be seen in pool A and pool B reactions of each sample. Only samples for which both pool A and pool B reactions have worked can be taken forward for sequencing

5. Sample Purification for PCR Products (optional)

40m

13

 # NOTE: This is an optional step but is **highly recommended** if primer dimers are detected in the PCR product

31m 30s

- Add AMPure XP **Bechman Coulter Catalog #**A63882 bead slurry to each sample in 1:1 volumetric ratio
- Incubate at Room temperature for 00:15:00 to allow DNA binding to beads
- Keep the sample tubes on magnetic stand for separation of beads bound to DNA
- Carefully remove supernatant without disturbing the beads
- Wash twice with 150 µL freshly prepared 80% Ethanol without disturbing the beads
- Keep for drying in Room temperature for 00:00:30 to 00:01:00 to remove excess ethanol. **NOTE:** Ensure that the beads do not dry completely.
- Remove tube from magnetic stand and add 25 µL of Nuclease Free Water (NFW). Mix the beads properly by pipetting
- Incubate for 00:15:00 at Room temperature to separate DNA from the beads
- Keep the tube on magnetic stand and wait for beads to separate out
- Recover the eluate (~20µl) containing DNA into a fresh tube

Precautions

- Always mix the beads only by pipetting or flicking to avoid DNA shear
- Always use freshly prepared 80% ethanol for washing
- Avoid carryover of beads while recovering the eluate in the last step

6. Oxford Nanopore Sequencing Steps Using SQK- RBK114.96 Kit

1h 30m

14 **# NOTE:** In the publication citing this protocol older reagents and R9.4 .1 flowcells were used. But since these reagents are discontinued by ONT, protocol mentioned below is updated with respect to new reagents compatible with R10.4.1 flowcells.

10m

- If bead purification (Step No. 13) was omitted, go directly to barcoding step go to step #14.1
- If bead purification of PCR product done, do QC using nanodrop and check ratios i.e. $A_{260/280}$ and $A_{260/230}$
- For the samples with good quality of DNA (as given in Step No. 5.2), Qubit readings are taken to determine DNA concentration go to step #5.1 .
- Atleast 50ng of DNA per sample is required for sequencing

14.1 **Barcoding**

9m

- Between 50 ng to 100 ng of each purified PCR product is taken in maximum volume of 9 µL (make up volume with NFW if needed) and 1 µL sequencing rapid barcode (RB01-96) provided in the kit is added

 # NOTE: If bead purification was not done, 9 µL of PCR product can be used directly in this step

- Mix properly by pipetting atleast 10 times
- Incubate at following conditions-

30 °C for 00:02:00 , then at 80 °C for 00:02:00 and then at 4 °C or On ice for 00:05:00

- Pool all the barcoded samples together in a single 1.5 mL microfuge tube
- All further steps are carried out on this pooled barcoded sample

14.2 **Purification of pooled barcoded sample**

31m 30s

- Add AMPure XP **Bechman Coulter Catalog #**A63882 bead slurry to each sample in 1:1 volumetric ratio

- Incubate at Room temperature for 00:15:00 to allow DNA binding to beads
- Keep the sample tubes on magnetic stand for separation of beads bound to DNA
- Carefully remove supernatant without disturbing the beads
- Wash twice with 1 mL freshly prepared 70% Ethanol without disturbing the beads
- Keep for drying in Room temperature for 00:00:30 to 00:01:00 to remove excess ethanol. **NOTE:** Ensure that the beads do not dry completely.
- Remove tube from magnetic stand and add 20 µL of **Elution Buffer (EB)** provided in the kit. Mix the beads properly by pipetting
- Incubate for 00:15:00 at Room temperature to separate DNA from the beads
- Keep the tube on magnetic stand and wait for beads to separate out
- Recover the eluate (~15µl) containing barcoded library into a fresh tube

Precautions

- Always mix the beads only by pipetting or flicking to avoid DNA shear
- Always use freshly prepared 70% ethanol for washing
- Avoid carryover of beads while recovering the eluate in the last step

 # NOTE: At this stage the barcoded library can be stored at 4 °C for 1 day and at -20 °C for longer duration (upto 1 month)

14.3 **Adapter ligation of barcoded library**

10m

- Take 11 µL of barcoded library and add 1 µL of diluted rapid adapter (1.5 µL Rapid Adapter (RA) + 3.5 µL Adapted Buffer (ADB))
- Incubate at 37 °C for 00:10:00 and immediately proceed with loading the library on the flowcell and sequencing

#NOTE: The sequencing library prepared is compatible with version 10 flowcells (FLOMIN_114) from **Oxford Nanopore Technology** (refer to manufacturer's website for latest update on flowcells and compatible kits).

14.4 **Flow cell priming**

15m

 # NOTE: This step can be performed during incubation period for adapter ligation (Step No. 14.3)

- Perform pore scan of the flow cell followed by **flowcell priming** as per ONT protocol

- Make priming mix by adding 30 µL Flow Cell Tether (FCT) in 1170 µL Flow Cell Flush (FCF) and mix by vortexing
- Remove waste from waste port if required (**# NOTE:** Make sure that the spot-on port and priming port are **CLOSED** during this step)
- Set 200 µL reading in 1000 µL pipette then **OPEN** priming port and suck air out by bringing pipette volume to 220 µL - 230 µL to avoid entry of air bubble while loading (**# NOTE:** Make sure that the spot-on port is **CLOSED** during this step)
- Add 800 µL of priming mix via the priming port. It is important to follow the method given **here** for this step. (**# NOTE:** Make sure that the spot-on port is **CLOSED** during this step)
- Incubate at room temperature for 00:05:00 to 00:10:00
- Do second priming of flow cell by adding 200 µL priming mix. It is important to follow the method given **here** for this step. (**# NOTE:** Make sure that the spot-on port is **OPENED** during this step)

Precautions

- While following priming steps, strictly avoid air bubble entry in the flowcell as this will result in pore loss
- Follow pipetting steps with care and check when to open or close spot-on port and priming port as per recommendation

14.5 **Sample preparation for loading**

2m

- Add 37.5 µL Sequencing Buffer (SB) and 25.5 µL Library Beads (LIB) to adapter ligated sequencing library
- **OPEN** spot-on port and add the prepared sample to spot-on port using 100 µL pipette. **NOTE:** Strictly avoid air bubble entry into spot-on port
- **CLOSE** both spot-on and priming port and remove any solution from waste port

Precautions

- While loading the sample, strictly avoid air bubble entry in the flowcell as it will result in pore loss
- Avoid leaving any liquid in the waste port after loading as this might block the ports and prevent reuse of flowcell if needed

14.6 **Starting the sequencing**

2m

- Open MinKNOW software
- Click on the left panel and select **Start**
- Then select **start sequencing** from the menu

- Select the flow cell position and enter your experiment name and sample ID
- Then click on kit selection and select-

  Rapid Barcoding Kit 96 V14 (SQK- RBK114.96) **Oxford Nanopore Technologies Catalog #**SQK- RBK114.96
- Move forward to select parameters as required and **start the sequencing**

15 **Data Analysis**

- In-house bash script named as **virAssem_2.sh** was developed for automated data analysis, accessible from github via **virAssem_2.git** link
- All steps involved in data analysis and the corresponding programs are described in **virAssem_2.git**

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