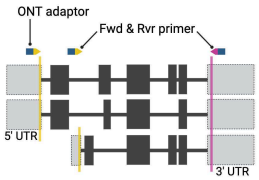
May 30, 2025 Version 2

A guide to long-range PCR for Nanopore sequencing V.2



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We use this protocol and it's working.

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Abstract

This protocol details how to design primers for a target gene and carry-out long-range PCR for subsequent Nanopore long-read amplicon sequencing (LSK114). It outlines the resources, tools and settings we use to amplify the entire annotated CDS of a gene, capturing as many alternative RNA isoforms as possible, and make these ready for a multiplexed Nanopore sequencing library. This protocol should reliably result in amplicons in the 1 - 11 kb range. Amplicons resulting from this protocol should be specific to transcripts of the gene of interest with minimal or no off-targets ensuring efficient sequencing of targets.

Image Attribution

Created in BioRender. De Paoli-Iseppi, R. (2024) https://BioRender.com/r06g873

Guidelines

RNA quality

- For best long-range PCR results for RNA isoform profiling the RNA quality (RIN) should be as high as possible (>7), although we have found RIN >6 works for many genes. The longer the isoforms of interest and thus the amplification length, the more important high RNA quality is.

Capturing RNA isoforms - how many primers do I need?

To ensure capture of known and unannotated RNA isoforms, you may need to design multiple different forward/reverse primers to cover alternate start/end sites. UCSC-GB tracks that can support primer design and selection include:

- Comprehensive Gene Annotation Set from GENCODE: All, non-coding, splice variants.
- Human mRNAs from GenBank.
- NCBI RefSeq genes.
- FANTOM5: CAGE reads.
- Human ESTs.

Using the above tracks can provide information and evidence to support design and placement of primers. For example, CAGE tags help identify bona fide transcriptional initiation sites. We suggest placing primers in the 5' and 3' UTR regions close to the ATG and STOP to minimise UTR sequence differences. If these areas are unsuitable for primers the first and last exon can also be used, however isoforms that use alternative exons will be missed. Avoid designing primers across exon-exon boundaries/junctions, as alternative use of either exon may result in amplification failure of specific RNA isoforms. Additionally primers that cross exon-exon boundaries may be able to prime and amplify solely from the 3' exon. This means you don't actually know what 5' exon was present and potentially what isoform was detected.
Note: it is a good idea to test primer pairs individually first to check amplification. E.g. individually test each forward primer with a single reverse: F1-R1 and F2-R1, prior to combing: F1 & F2 with R1.

Taq polymerase & PCR optimisation

There are several DNA polymerases available that are capable of amplifying long products. In some cases it is important to try a different polymerase if one fails to amplify the expected product. Selection of a suitable polymerase can depend on several factors including reaction speed (min/kb), cost, amplicon size and fidelity. In most cases we suggest using test RNA/cDNA first to optimise PCR conditions, considering overall cycles, annealing temperature (test gradient) and 2- or 3-step protocols depending on primer lengths.

Generally, PCR cycles should be kept to a minimum to reduce PCR bias and artifacts, however there needs to be enough PCR product post bead clean-up to ensure reliable ONT barcoding. We commonly barcode 1 ng of PCR product, so the yield required is low, helping keep PCR cycles down.

Materials

We used the following reagents and equipment for Nanopore (ONT) long-range PCR, library preparation and sequencing.

Order **primers** with ONT Universal Primers (UP) attached:

- **Scale** (µmole): .025
- **Purification**: desalt
- **Format**: liquid/dry – as preferred.
- **Concentration** if in liquid: [M] 100 micromolar (µM)
- Prepare **dilutions** for use at [M] 10 micromolar (µM), (e.g. 90 µL NFW + 10 µL primer stock).

Options tested for long-range PCR taq polymerase

LongAmp Taq 2X Master Mix - 100 rxns **New England Biolabs** **Catalog #**M0287S

PrimeSTAR GXL DNA Polymerase **Catalog #**R050A

Platinum™ SuperFi II PCR Master Mix **Invitrogen - Thermo Fisher** **Catalog #**12368050

Gel electrophoresis

GeneRuler 1 kb DNA Ladder, ready-to-use **Thermo Fisher** **Catalog #**SM0314

GelGreen™ Nucleic Acid Stain Gel Stain, 10,000X in Water **Gold Biotechnology** **Catalog #**G-745

Library prep and sequencing

Agencourt AMPure XP **Beckman Coulter** **Catalog #**A63880

Equipment	
MinION	NAME
Sequencer	TYPE
Oxford Nanopore Technologies	BRAND
MinION 1B / MinION 1C	SKU

Equipment

Flongle Flow Cell (R10.4.1)	NAME
Flow cell	TYPE
Oxford Nanopore Technologies	BRAND
FLO-FLG114	SKU
https://store.nanoporetech.com/flongle-flow-cell-pack-r10-4-1.html	LINK

Equipment

MinION/GridION Flow Cell (R10.4.1)	NAME
Flow cell	TYPE
Oxford Nanopore Technologies	BRAND
FLO-MIN114	SKU
https://store.nanoporetech.com/flow-cell-r10-4-1.html	LINK

Protocol materials

- RNeasy Lipid Tissue Mini Kit (50) **Qiagen** **Catalog #**74804
- Maxima H Minus Reverse Transcriptase **Thermo Fisher Scientific** **Catalog #**#EP0741
- GeneRuler 1 kb DNA Ladder, ready-to-use **Thermo Fisher** **Catalog #**SM0314
- GelGreen™ Nucleic Acid Stain Gel Stain, 10,000X in Water **Gold Biotechnology** **Catalog #**G-745
- Agencourt AMPure XP **Beckman Coulter** **Catalog #**A63880
- Platinum™ SuperFi II PCR Master Mix **Invitrogen - Thermo Fisher** **Catalog #**12368050
- LongAmp Taq 2X Master Mix - 100 rxns **New England Biolabs** **Catalog #**M0287S
- PrimeSTAR GXL DNA Polymerase **Catalog #**R050A

Safety warnings

All steps should be carried out by trained personnel in a certified PC2 laboratory and follow established SOPs, RAs and have SDSs available for all reagents used.

Ethics statement

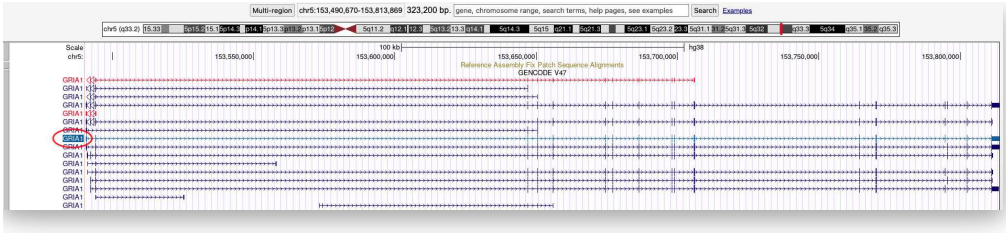
The Human Research Ethics Committee of the University of Melbourne gave ethical approval for this work: #12457 and #28304.

Before start

- QC total RNA before cDNA conversion. RNA quality/integrity should be as high as possible (RIN > 7).
- Read Guidelines for additional primer and PCR design tips.

Retrieve CDS of target isoform - UCSC Genome Browser

1 Search for a gene of interest in UCSC Genome Browser[1] and select the main isoform – usually
highlighted as indicated below:



UCSC Genome Browser screenshot of *GRIA1* highlighting the main isoform.

- Details on position, size, coding exon count and strand can be found here.
- Further details including Biotype, Transcript Support Level (TSL) and APPRIS classification can be found under the GENCODE Transcript Annotation.

Human Gene GRIA1 (ENST00000285900.10) from GENCODE V47

Description: glutamate ionotropic receptor AMPA type subunit 1, transcript variant 10 (from RefSeq NM_001364165.2)
Gencode Transcript: ENST00000285900.10
Gencode Gene: ENSG00000155511.19
Transcript (Including UTRs)
Position: hg38 chr5:153,490,670-153,813,869 **Size:** 323,200 **Total Exon Count:** 16 **Strand:** +
Coding Region
Position: hg38 chr5:153,490,889-153,811,225 **Size:** 320,337 **Coding Exon Count:** 16

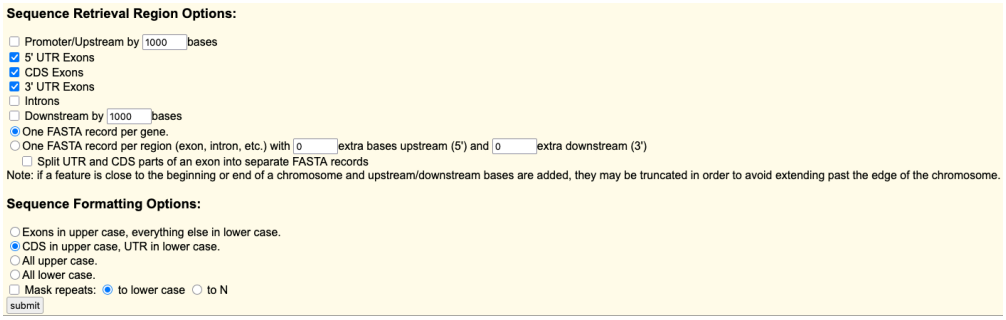
GRIA1 main isoform details.

GENCODE V46 Transcript Annotation ENST00000285900.10 (GRIA1)

	Transcript	Gene
GENCODE id	ENST00000285900.10	ENSG00000155511.19
Protein id	ENSP00000285900.4	
HAVANA manual id	OTTHUMT00000252456.4	OTTHUMG00000130148.7
Position	chr5:153490670-153813869	chr5:153489615-153813869
Strand	+	
Biotype	protein_coding	protein_coding
Annotation Level	manual (2)	
Annotation Method	manual & automatic	manual & automatic
Transcription Support Level	tsl1	
HGNC gene information	HGNC:4571	
GeneCards	GRIA1	
CCDS	CCDS4322.1	
Transcript rank	1	
APPRIS	PRINCIPAL:3	GRIA1

GRIA1 main isoform additional Transcript Annotation details.

1.1 Select ‘Genomic Sequence’ and use the settings shown below to retrieve the sequence including 5′ and 3′ UTR regions. This sequence can then be copied into Primer3Plus[2] and used for primer pair selection.



Settings for main isoform CDS retrieval.

Design primers using Primer3Plus

2 In Primer3Plus[2] (v3.3.0, server: default), copy target sequence into the space and mark the coding region (CAPITALS) with [] to target this region. Press the 'Regions from Seq.' button to apply.

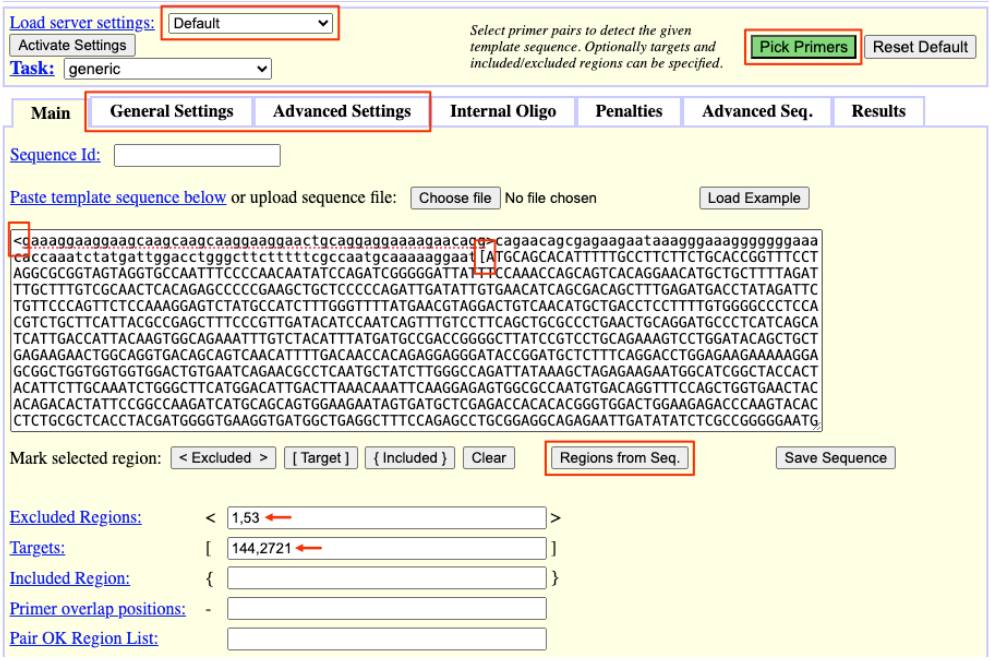
- Primers should be designed in the 5′ and 3′ UTRs (lower case) and close to start and stop codons where possible.
- Exclude UTR areas far from start and stop codons with <> in the sequence.
- Avoid repetitive regions.
- Avoid designing primers across exon-exon boundaries.

General Settings

- Ensure **Product Size Ranges** includes long PCR products e.g. 1000-15000.
- Primer **size**: min:18, opt:20, max:27.
- Primer **Tm**: min:57, opt:60, max:63. (Aim for a max of 2°C difference).
- Primer **GC%**: min:20, max:80.
- Turn on **Mispriming/Repeat Library** option to appropriate species (e.g. HUMAN).

Advanced Settings

- **Max Poly-X**: 3 – 4.
- **CG Clamp**: 1 – 2. If possible, try primer selection with this set to 2 first and reduce if no primers are found or adjust window selection.

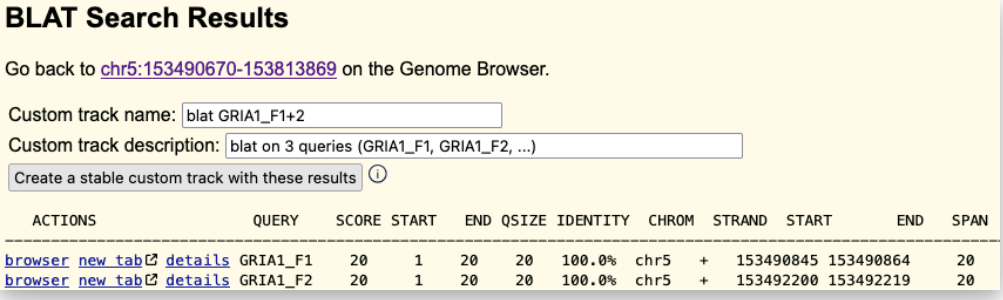


Example settings for Primer3Plus.

Check primers for specificity & off-target amplification

3 Check that your designed primers are unique by using UCSC tool **BLAT**[3]. "BLAT on DNA is designed to quickly find sequences of 95% and greater similarity of length 25 bases or more."

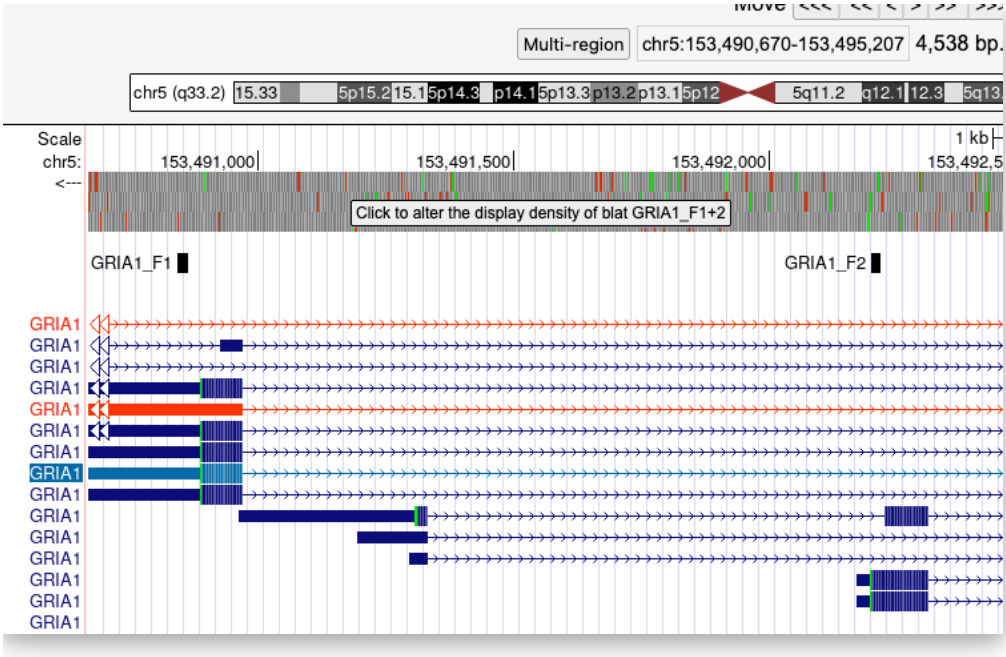
Copy and paste or upload your primer sequences (min: 20 bp) without the universal primer and click submit e.g.:
>GRIA1_F1
TATGATTGGACCTGGGCTTC
>GRIA1_F2
TGTGCTGCAGTACCCATCTC



BLAT search results for *GRIA1* indicating match score, identity, chromosome, strand and start/end positions.

A custom 'track' can be made so that you can view primer position in UCSC.

- Click "build a custom track with these results".
- Click "Track controls" > Submit and navigate to gene of interest on UCSC GB.



Custom track showing multiple *GRIA1* forward primers designed using this protocol.

3.1 Perform PCR using UCSC tool: **In-silico PCR**[4].

"In-silico PCR searches a sequence database with a pair of PCR primers."

- Max Product Size: 10000
- Target: GENCODE Genes
- Copy your forward and reverse primer pair (without universal primers) into the spaces > Submit.
- This will return exact matched 'amplified' sequences, check the ENST number against those in UCSC-GB to see which transcripts you aim to capture.

To design additional primers go to step #1

ONT Universal Primers (UP)

4 When ordering primers for Nanopore long-read sequencing, add universal sequences for tailing PCR Primers as below. Testing without universal primers first may be necessary as they can reduce efficiency.

The first round of PCR amplification requires tailed primers to be used which carry these sequences:

- 5′ TTTCTGTTGGTGCTGATATTGC-[project-specific forward primer sequence] 3′
- 5′ ACTTGCCTGTCGCTCTATCTTC-[project-specific reverse primer sequence] 3′

Standard example - *GRIA1* (2.7 kb)

5 Using the steps above, we designed primers for Glutamate Ionotropic Receptor AMPA Type Subunit 1 (*GRIA1*) encompassing a 2787 bp coding region and two supported start sites.

Primers

>GRIA1_F1

TATGATTGGACCTGGGCTTC

20 bp, 59.9 °C, GC:50%

>GRIA1_F2

TGTGCTGCAGTACCCATCTC

20 bp, 59.9 °C, GC:55%

>GRIA1_R1

AAGGGGTCTCCATCTGCTC

19 bp, 59.2 °C, GC:57.9%

5.1 Isolate high-quality total RNA from sample following manufacturers' instructions.

Safety information

QIAzol (and equivalents) are toxic. Personnel should be trained in the SOP and appropriate PPE (gloves, eye protection) must be worn when handling this chemical.

RNeasy Lipid Tissue Mini Kit (50) **Qiagen Catalog #**74804

5.2 Generate cDNA using 1 µg total RNA and follow manufacturers' instructions.

Maxima H Minus Reverse Transcriptase **Thermo Fisher Scientific Catalog #**#EP0741

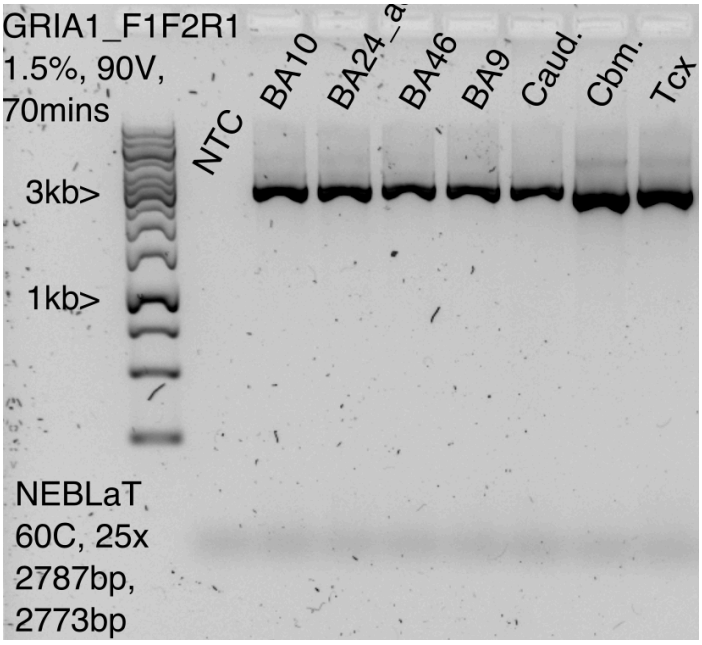
5.3 **Optional**: Use test or 'non-critical' samples/cDNA to test the below PCR conditions including anneal temperature, extension and cycles. Cycles should be kept to a minimum to reduce PCR bias.

5.4 Prepare 1st round 25 µL PCR reaction for *GRIA1* using a standard 3-step PCR approach - 25x cycles ***with*** tailed (ONT - UP) gene specific primers. Annealing/extension 60 °C for 00:03:00 .

LongAmp Taq 2X Master Mix - 100 rxns **New England Biolabs Catalog #**M0287S

Component	Final conc.	25 µL rxn
Fwd Primer (UP)	0.4 µM	1 µL
Rvr Primer (UP)	0.4 µM	1 µL
LongAmp Taq 2X	1X	12.5 µL
Template cDNA	-	1 µL
NFW	-	9.5 µL

5.5 Check PCR amplicon e.g. gel electrophoresis/TapeStation. Gel: 1.5%, 90V, 01:10:00 .



Example 1st round amplification of GRIA1 in post-mortem human brain tissues using multiple forward (F1, F2) primers. These amplicons are ready for clean-up and barcoding for ONT library preparation. *No template control (NTC), Brodmans area (BA), caudate (Caud), cerebellum (Cbm), temporal cortex (Tcx).*

GeneRuler 1 kb DNA Ladder, ready-to-use **Thermo Fisher Catalog #**SM0314

GelGreen™ Nucleic Acid Stain Gel Stain, 10,000X in Water **Gold Biotechnology Catalog #**G-745

5.6 Clean-up 1st round PCR: add 0.5X 12.5 µL AMPure XP beads to PCR, follow clean-up procedure and elute in 10 µL NFW.

Agencourt AMPure XP **Beckman Coulter Catalog #**A63880

5.7 Cleaned-up amplicon(s) may now be used for ONT barcoding and multiplexed long-read sequencing following LSK114 library preparation.
https://nanoporetech.com/document/ligation-sequencing-gdna-pcr-barcoding-sqk-lsk114-with-exp

Technical example (long) - *CSMD1* (10.8 kb)

4h

6 The schizophrenia risk gene CUB and sushi multiple domains 1 (*CSMD1*) has the longest CDS we amplified at ~10,838 nt, encompassing 70 coding exons[5,6].

1h 21m

Primers

>CSMD1_F1

CCCTCGGGTGATTATTTGG

19 bp, 60.1 °C, GC:52.6%

>CSMD1_R1

CACTGCTTGTCCATCAGAGG

20 bp, 59.4 °C, GC:55%

6.1 go to step #5.1

6.2 go to step #5.2

6.3 Prepare 1st round 20 µL PCR reaction for *CSMD1* using a 2-step PCR approach - 5x cycles with ***no*** ONT Universal Primers (UP). Annealing/extension 72 °C for 00:05:30 .

Platinum™ SuperFi II PCR Master Mix **Invitrogen - Thermo Fisher Catalog #**12368050

Component	Final conc.	20 µL rxn
Fwd Primer (no UP)	0.5 µM	1 µL
Rvr Primer (no UP)	0.5 µM	1 µL
2x SuperFi II PCR MM	1X	10 µL
Template cDNA	-	1 µL
NFW	-	7 µL

6.4 Clean-up 1st round PCR: add 0.45X 9 µL AMPure XP beads to PCR, follow clean-up procedure and elute in 8 µL NFW.

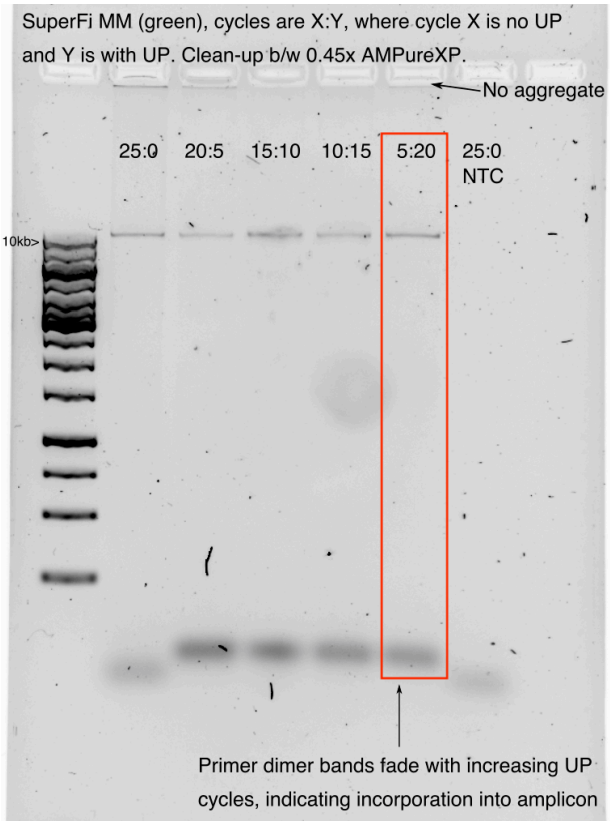
Agencourt AMPure XP **Beckman Coulter Catalog #**A63880

6.5 Prepare 2nd round 20 µL PCR reaction for *CSMD1* (2-step PCR)- 20x cycles ***with*** ONT Universal Primers attached. Annealing/extension 72 °C for 00:05:30 .

Platinum™ SuperFi II PCR Master Mix **Invitrogen - Thermo Fisher Catalog #**12368050

Component	Final conc.	20 µL rxn
Fwd Primer (UP)	0.5 µM	1 µL
Rvr Primer (UP)	0.5 µM	1 µL
2x SuperFi II PCR MM	1X	10 µL
Purified 1st rnd PCR	-	8 µL

6.6 Check PCR amplicon e.g. gel electrophoresis/TapeStation. Gel: 1.5%, 90V, 01:10:00 .



Primer optimisation result for *CSMD1* amplicon. Red box indicates optimal 2-step PCR protocol to result in product incorporated with ONT universal primer tags for subsequent barcoding and library preparation.

GeneRuler 1 kb DNA Ladder, ready-to-use **Thermo Fisher Catalog #**SM0314

GelGreen™ Nucleic Acid Stain Gel Stain, 10,000X in Water **Gold Biotechnology Catalog #**G-745

6.7 Cleaned-up amplicon(s) may now be used for ONT barcoding and multiplexed long-read sequencing following LSK114 library preparation.
https://nanoporetech.com/document/ligation-sequencing-gdna-pcr-barcoding-sqk-lsk114-with-exp

Protocol references

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3. Kent WJ. **BLAT - the BLAST-like alignment tool**. Genome Res. 2002 Apr;12(4):656-64. PMID: 11932250
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5. M. B. Clark, T. Wrzesinski, A. B. Garcia, N. A. L. Hall, J. E. Kleinman, T. Hyde, et al. Long-read sequencing reveals the complex splicing profile of the psychiatric risk gene CACNA1C in human brain. Molecular Psychiatry. 2020. Vol. 25 Issue 1 Pages 37-47. DOI: 10.1038/s41380-019-0583-1.
6. R. D. Paoli-Iseppi, S. Joshi, J. Gleeson, Y. D. Joseph Prawer, Y. You, R. Agarwal, et al. Long-read sequencing reveals the RNA isoform repertoire of neuropsychiatric risk genes in human brain. medRxiv. 2024. DOI: 10.1101/2024.02.22.24303189.

Useful links
UCSC Genome Browser
https://genome.ucsc.edu/cgi-bin/hgGateway
BLAT
https://genome.ucsc.edu/cgi-bin/hgBlat?hgsid=781293703_jTewuFFIMrnHihwxk6pqzBPAkLTb&command=start
In-silico PCR
https://genome.ucsc.edu/cgi-bin/hgPcr?hgsid=719921545_TYRWW40X6Mz5ecHz7ayAt1ElpziP
Primer3Plus
https://www.primer3plus.com/
Reverse Complement
https://www.bioinformatics.org/sms/rev_comp.html

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