

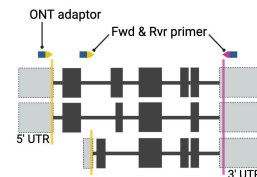
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Version 1

A guide to long-range PCR for Nanopore sequencing V.1

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Protocol status: Working

We use this protocol and it's working.

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Grant ID: GNT1196841

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

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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 combining: 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 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

- ✕ Maxima H Minus Reverse Transcriptase **Thermo Fisher Scientific Catalog ##EP0741**
- ✕ LongAmp Taq 2X Master Mix - 100 rxns **New England Biolabs Catalog #M0287S**
- ✕ Agencourt AMPure XP **Beckman Coulter Catalog #A63880**
- ✕ Platinum™ SuperFi II PCR Master Mix **Invitrogen - Thermo Fisher Catalog #12368050**
- ✕ 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**
- ✕ PrimeSTAR GXL DNA Polymerase **Catalog #R050A**
- ✕ RNeasy Lipid Tissue Mini Kit (50) **Qiagen Catalog #74804**

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

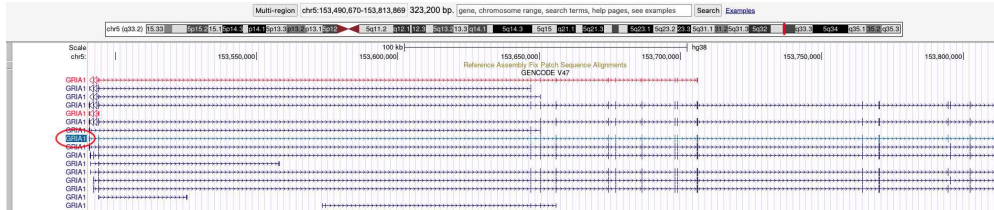
The Human Research Ethics Committee of the University of Melbourne gave ethical approval for this work: #12457 and #28304.

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 Browser¹ 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)
Genecode Transcript: ENST00000285900.10
Genecode 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 Primer3Plus² and used for primer pair selection.



Sequence Retrieval Region Options:

☐ Promoter/Upstream by 1000 bases

☒ 5' UTR Exons

☒ CDS Exons

☒ 3' UTR Exons

☒ Introns

☐ Downstream by 1000 bases

☒ One FASTA record per gene.

☐ One FASTA record per region (exon, intron, etc.) with 0 extra bases upstream (5') and 0 extra downstream (3')

☐ Split UTR and CDS parts of an exon into separate FASTA records

Note: if a feature is close to the beginning or end of a chromosome and upstream/downstream bases are added, they may be truncated in order to avoid extending past the edge of the chromosome.

Sequence Formatting Options:

☐ Exons in upper case, everything else in lower case.

☒ CDS in upper case, UTR in lower case.

☐ All upper case.

☐ All lower case.

☐ Mask repeats: ☒ to lower case ☐ to N

Settings for main isoform CDS retrieval.

Design primers using Primer3Plus

- 2 In Primer3Plus², copy target sequence into the space and mark the coding region (CAPITALS) with [] to target this region.
- 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 <>.
 - Avoid repetitive regions.
 - Avoid designing primers across exon-exon boundaries.



General Settings

- Adjust **Product Size Ranges** to include long PCR products e.g. 1000-15000.
- Primer **size**: min:18, opt:20, max:27.
- Primer **Tm**: min:57, opt:60, max:63.
- 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.

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

Go back to [chr5:153490670-153813869](#) on the Genome Browser.

Custom track name:

Custom track description:

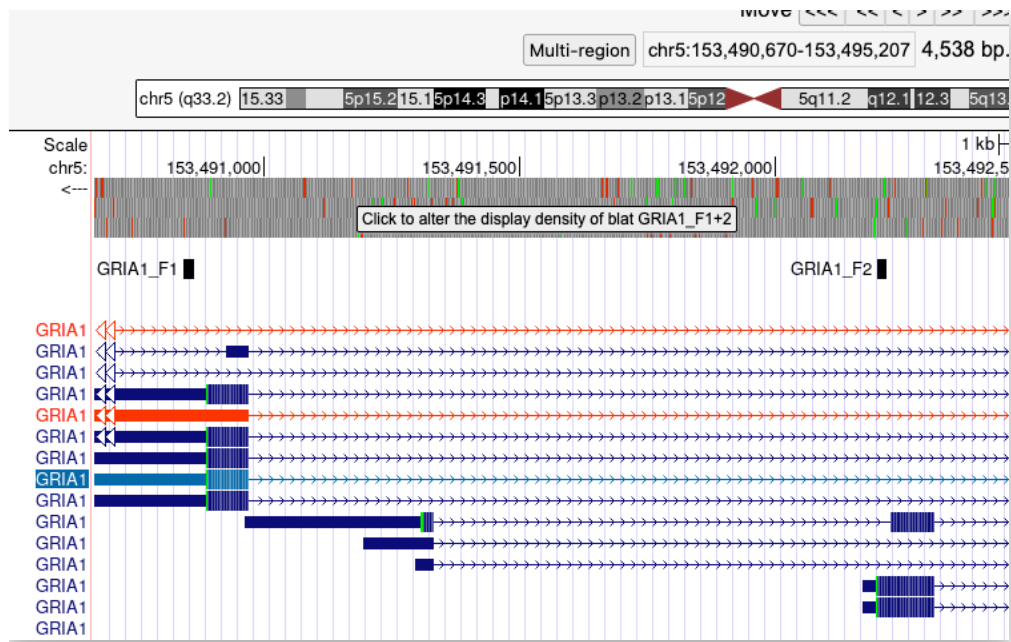
[Create a stable custom track with these results](#) ⓘ

ACTIONS	QUERY	SCORE	START	END	QSIZE	IDENTITY	CHROM	STRAND	START	END	SPAN
browser new tab details	GRIA1_F1	20	1	20	20	100.0%	chr5	+	153490845	153490864	20
browser new tab details	GRIA1_F2	20	1	20	20	100.0%	chr5	+	153492200	153492219	20

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

- 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' TTTCTGTTGGTGTGATATTGC-[project-specific forward primer sequence] 3'
- 5' ACTTGCTGTGCTCTATCTTC-[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.

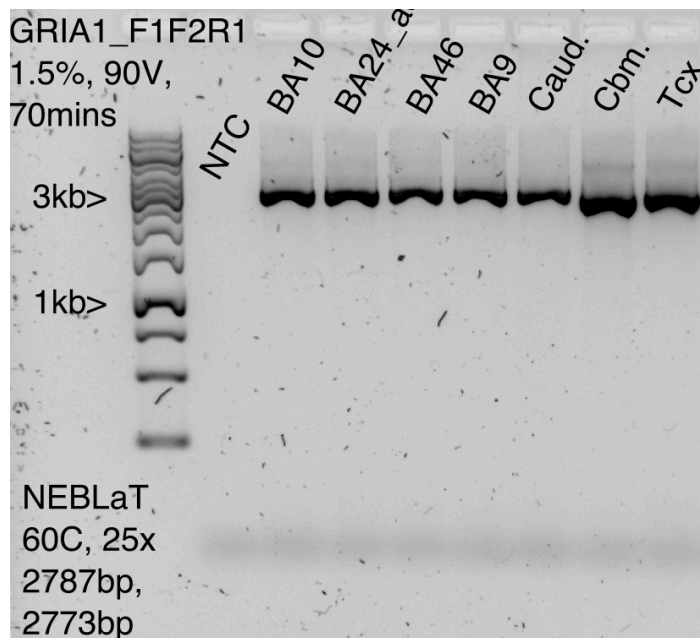


- 5.4 Prepare 1st round 25 µL PCR reaction for *GRIA1* using a standard 3-step PCR approach - 25x cycles **with** ONT Universal Primers (UP). Annealing/extension 60 °C for 00:03:00 .

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

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

- 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 μ L AMPure XP beads to PCR, follow clean-up procedure and elute in 10 μ L 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

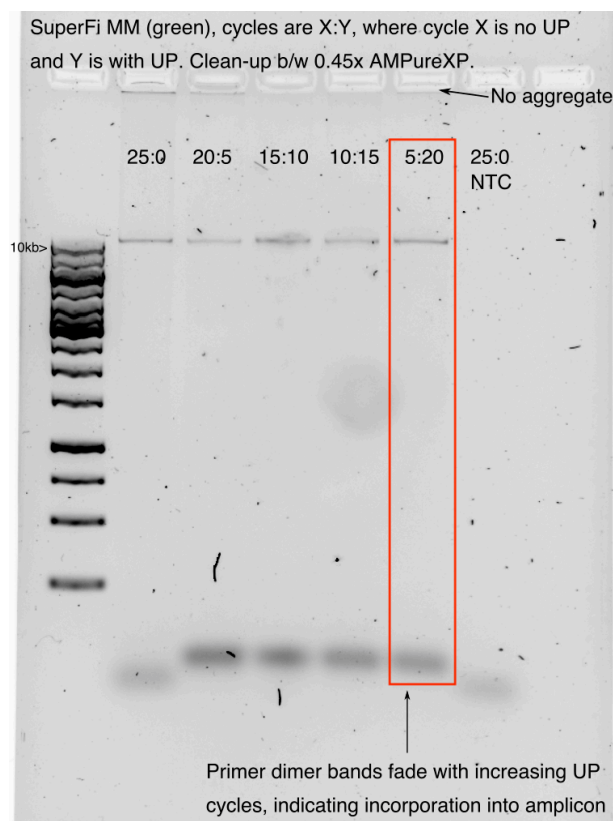
- 6.5 Prepare 2nd round  20 µL PCR reaction for *CSMD1* (2-step PCR)- 20x cycles **with** ONT Universal Primers attached. Annealing/extension  72 °C for  00:05:30 .



 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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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

<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>

Reverse Complement

https://www.bioinformatics.org/sms/rev_comp.html

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