

Multiplex PCR explainer

Sequencing viral genomes: There are a variety of approaches to sequence virus genomes including metagenomics, which is ideal for virus discovery because it is an untargeted method (e.g. generated first SARS-CoV-2 genome). However, untargeted methods are usually less suited to genomic surveillance where sequencing large numbers of isolates becomes prohibitively expensive. An alternative approach is PCR-based target enrichment which produces amplicons that span the viral genome, i.e. the ARTIC multiplex PCR method. This method has been widely used for a range of viruses, including SARS-CoV-2, rabies virus, yellow fever, polio etc. It has proven robust to a large range of input titres, is highly specific and scalable to hundreds of genomes. In an outbreak situation in which isolates are highly related, and low cost per sample and rapid turnaround time are required, PCR is particularly suitable.

Key reference: Quick J, *et al.* Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples. *Nat Protoc.* 2017 Jun;12(6):1261-1276. doi: 10.1038/nprot.2017.066. Epub 2017 May 24. PMID: 28538739; PMCID: PMC5902022.

Multiplex Polymerase Chain Reaction (mPCR): A technique that allows the simultaneous amplification of multiple target DNA sequences in a single reaction tube. This offers increased efficiency and throughput in various molecular biology applications. In mPCR, multiple pairs of primers are designed to target different regions of DNA in the same reaction. Each primer pair specifically binds to its respective target sequence.

Rabies virus (RABV) mPCR primer design: Regional primer schemes have been developed for active RABV genomic surveillance projects in Africa, Asia and South America. Primers were designed against sets of representative reference genomes found in publicly available archives like NCBI GenBank, using a primer design tool called Primal Scheme (<https://primalscheme.com/>). Due to the divergence of different RABV lineages, it is necessary to create separate schemes to capture each set of diversity in a geographic region, however multiple schemes can be combined (and tested) and efforts can be made to develop degenerate primer sets to capture a broader diversity. But, ultimately PCR is *specific* therefore if the diversity is high it may be better to consider alternative approaches like probe based capture.

Key primer design and PCR conditions:

These design features and reaction conditions ensure high sensitivity and reduces competitive inhibition (between primers)

- Low individual primer concentrations
- Primers have high primer annealing temperatures within a specific range (65-68 °C)
- Long annealing times
- Allows PCR to be performed as a 2-step protocol (95 °C denaturation, 65 °C combined annealing and extension)
- Alternate target genome regions are assigned to one of two primer pools, so that neighboring amplicons do not overlap within the same pool (which would result in a short overlap product being generated preferentially).

- Strategy of targeting short amplicon lengths (400bp) to help deal with potentially degraded rabies samples, which enhances the likelihood of successful amplification and whole genome capture

Expected results/Learning outcomes:

- Concentrations measured at the next station (Quantus) should match your pre-recorded Quantus measurement (+/- 3ng/ul)
- Negative control should have no reading (either <1ng/ul or unreadable)
- Good understanding of multiplex PCR and applied use
- Emphasises importance of quality control, good lab practice and dealing with batches of samples

Table 1. Reaction set-up for multiplex PCR

	<i>Per reaction</i>			<i>Mastermix</i>	
Reagent	Pool A (μl)	Pool B (μl)	No of reactions (+ 10% excess)	Pool A	Pool B
Q5 Hot Start High-Fidelity 2X Master Mix	6.25	6.25			
Primer pool A	0.75	0			
Primer pool B	0	0.75			
Nuclease free water	3	3			