Development of a High-Throughput SARS-CoV-2 Strain-Typing Assay

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PURPOSE

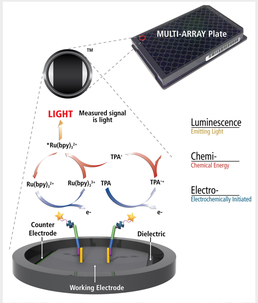
The current COVID-19 pandemic has highlighted the need for high-throughput and cost-effective methods for assessing mutations, including single nucleotide polymorphisms (SNPs), in SARS-CoV-2 genomic RNA. Understanding the mutations in a given strain is important for several reasons: (1) SNP-driven protein changes are critical determinants of viral pathogenesis and transmission, (2) monitoring SNPs is useful for strain typing and the epidemiological assessment of transmission patterns, and (3) showing the effectiveness of clinical treatment and vaccine strategies against multiple viral strains or strains with mutations known to impact pathogenicity will be essential. One example of the utility of SNP genotyping is with the differentiation of the S strain type from the more aggressive L strain type of SARS-CoV-2 by two nucleic acid substitutions (8782 C>T and 28144 T>C), with both types persisting in many strains to this day. Another is the D614G change in the spike protein that results from an A>G mutation at site 23403. Furthermore, a G>T change at site 11083 has been associated with a shift towards asymptomatic infections. All four of these mutations have also been helpful in differentiation of strain types or clades of SARS-CoV-2.

Studies that track specific mutations rely on expensive and low-throughput, whole-viral genome-sequencing strategies. We sought to develop a multiplex test to rapidly distinguish between different strain types and mutations of SARS-CoV-2 based on single nucleotide polymorphisms (SNPs) in the viral genome.

METHODS

MSD Technology:

MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT® microplates.



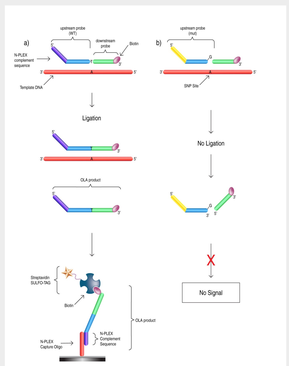
Benefits of Electrochemiluminescence Technology

- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- Carbon electrode surface has 10X greater binding capacity than polystyrene wells.
- Surface coatings can be customized.

SNP Detection with OLA and the N-PLEX® Platform:

The oligonucleotide ligation assay (OLA) uses probes that were specific for DNA sequences upstream and downstream of the SNP of interest, with only exact matches at the SNP site allowing for the ligation of the two probes. The ligated probes were hybridized to spot-specific capture oligos on N-PLEX plates to allow for detection.

- Each well in an N-PLEX 96-well plate has 10 unique capture oligos attached to the surface of the plate, allowing for the detection of up to five SNPs per well.
- Three probes were needed per target: a biotinylated (downstream) probe and two (upstream) probes that recognized either polymorphic base and contained a sequence complementary to a specific capture oligo.
- Taq DNA ligase was used to join upstream and downstream probes that aligned correctly on a given DNA sample. Fragments of unmodified template complements were added to prevent bridging of unligated probes.
- OLA products (from synthetic oligos or RT-PCR products from RNA samples) were hybridized to the appropriate capture oligo on the N-PLEX plate, bound by SULFO-TAG labeled streptavidin, and analyzed using an MSD® instrument.



- Swabs from COVID-19+ patients were obtained from BOCA Biolistics, and RNA was extracted using the MagMax Viral/Pathogen Ultra Nucleic Acid Isolation Kit.
- RT-PCR was conducted with site-specific primers using TaqPath 1-step RT-qPCR Master Mix.
- Reference strain RNAs were obtained from BEI Resources, NIAID, NIH: Genomic RNA from SARS-Related Coronavirus 2, Isolate USA-WA1/2020, NR-52285, GenBank MN985325, deposited by the Centers for Disease Control and Prevention; Genomic RNA from SARS-Related Coronavirus 2, Isolate Hong Kong/VM20001061/2020, NR-52388, GenBank MT547814, deposited by the University of Hong Kong.

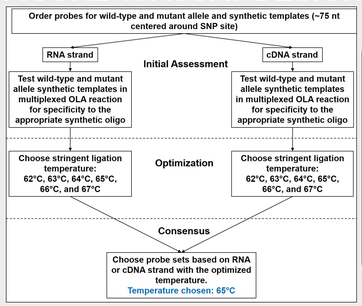
RESULTS

TABLE 1: SELECTED SNPs

Nucleic Acid Change	Site in viral RNA	Functional Outcome
C>T	8782	One of two differentiating alleles for S strain type, less aggressive than L strain type
G>T	11083	Associated with asymptomatic infections, included in V clade
A>G	23403	Higher transmission rate and more pathogenic, included in G clade
T>C	28144	One of two differentiating alleles for S strain type, less aggressive than L strain type

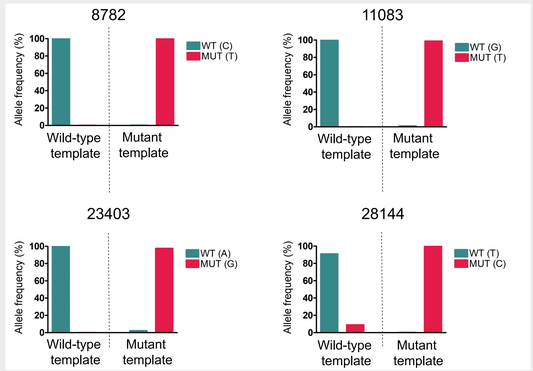
The SNPs above were chosen based on their prevalence, suspected functional outcome on the virus and/or host, as well as their ability to be used in strain or clade differentiation. Furthermore, making new SNP assays for the N-PLEX platform is straightforward and rapid.

FIGURE 1: ASSAY DESIGN/OPTIMIZATION



Optimized Protocol

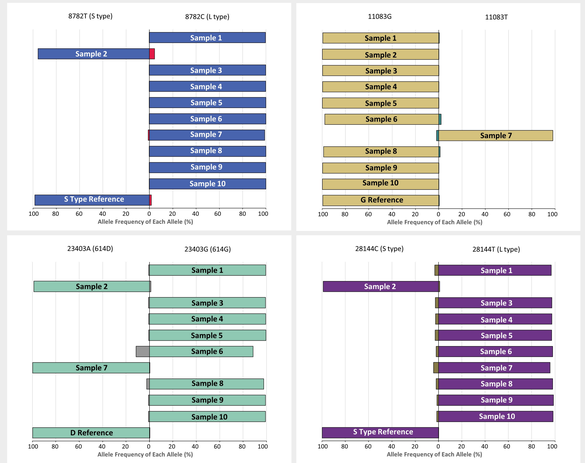
1. Perform OLA on synthetic oligos with multiplexed probe sets and Taq DNA Ligase (2 minutes at 95 °C and 30 cycles of 30 sec at 95 °C and 2 minutes at 65 °C). Block N-PLEX plate for 30 minutes at 37 °C during OLA cycling.
2. Wash the plate and add OLA product in hybridization buffer (50 µL per well) to the plate. Incubate for 1 hour at 37 °C.
3. Wash the plate and add detection solution (50 µL per well). Incubate 30 minutes at 37 °C.
4. Wash the plate and add read buffer (150 µL per well). Analyze with MSD instrument.



Synthetic oligo templates show high specificity for the appropriate allele using the optimized temperature condition of 65 °C, respectively. WT = Wild-type allele, MUT = Mutant allele. **All four assays fit well within our allele frequency guidelines for calling SNPs: Homozygous ≥ 80% and Not Present ≤ 20%.**

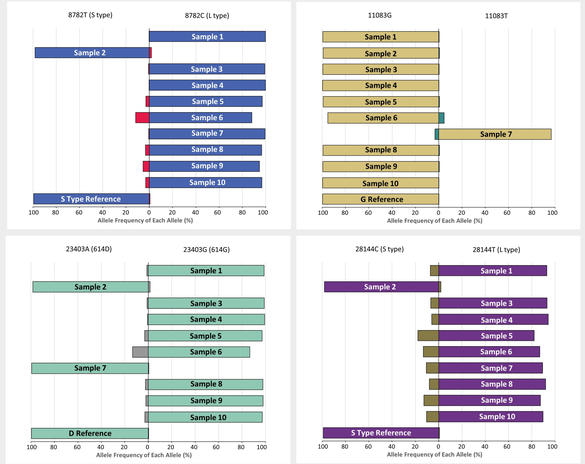
RESULTS

FIGURE 2: SINGLEPLEX ASSAYS



RNA was extracted from 10 nasopharyngeal swabs from COVID-19+ patients and amplified with site specific primers via RT-PCR. The optimized protocol (see Figure 1) was used to run OLA and N-PLEX on each of the 4 target SNPs in singleplex. There is a high prevalence of L strain types (8782C & 28144T) that also have the 614G mutation. Only one strain had the asymptomatic allele (11083T). Results were compared with a fully sequenced reference strain, with all results matching the published sequences for these strains. All samples fit in our allele frequency guidelines for SNP calling.

FIGURE 3: MULTIPLEX ASSAYS



RNA that had been extracted from the 10 nasopharyngeal swabs from COVID-19+ patients was amplified with multiplexed site specific primers via RT-PCR. The optimized protocol (see Figure 1) was used to run OLA and N-PLEX on each of the 4 target SNPs in multiplex. The resulting SNP calls in the multiplex assay are consistent with the singleplex assays. All samples fit in our allele frequency guidelines for SNP calling.

RESULTS

TABLE 2: OLA REPRODUCIBILITY

Site	WT samples (WT allele)				MUT samples (MUT allele)			
	Mean	STD	CV	Total N	Mean	STD	CV	Total N
8782	97.8	3.1	0.03	54	98.6	0.3	0.003	6
11083	99.3	2.8	0.03	54	98.1	1.0	0.01	6
23403	99.2	0.4	0.004	12	100.3	5.4	0.05	48
28144	96.3	10.0	0.1	54	98.7	0.8	0.01	6

Multiplexed RT-PCR products from the samples run in Figure 3 were subjected to 3 separate multiplexed OLA reactions on 3 different days to determine the allele frequency reproducibility from the 4 SNP assays. The mean, standard deviation (STD), CV, and total number (N) of readings are shown for those samples that either had the WT or mutant (MUT) allele for the given SNP assay. All assays show good allele frequency reproducibility. Also, the SNP calls for all 10 samples and the reference were consistent between runs for all 3 experiments.

CONCLUSIONS

- **Low off-target signals (and frequencies) allow for easy allele discrimination between strains.**
- **Results are highly reproducible.**
- **The ability to call SNPs is not influenced by the host RNA, making SNP determinations straightforward.**
- **Assay time after RNA extraction is ~6 hours for a full 96-well plate.**
- **96-well format allows for up to 480 SNP determinations in a single plate (up to 5 SNP determinations per well).**
- **Assays can be designed rapidly, with a turnaround time of about 3 weeks for a new SNP assay.**
- **The N-PLEX SNP assays offers a high-throughput alternative to sequencing for the determination of specific SNPs in SARS-CoV-2 strains.**

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