Syndromic testing for medium throughput in one workflow: flexible molecular analysis with the MODAPLEX platform

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Introduction

Due to the COVID-19 pandemic, the demand for molecular diagnostics in diagnosing, treating, and monitoring infectious diseases has drastically increased. With different strains of SARS-CoV2 appearing, and other viral pneumonia associated pathogens expressing similar symptoms, clinicians need a rapid and accurate method to differentiate between various pathogens [1]. Hence, the clinical need for syndromic testing grew exponentially.

To address this issue, we present the MODAPLEX platform as a tool to simplify syndromic testing [2]. We successfully consolidated several established qPCR assays for infectious disease into one robust qPCR multiplex assay on the MODAPLEX platform. The transfer of assays was achievable with only minor modification of the probes. The MODAPLEX merges PCR and capillary gel electrophoresis (CE), enabling target discrimination and semi-quantitative analysis of all crucial targets in one multiplex reaction. During PCR amplification, fluorescently labelled amplicons and/or probe-fragments of the ongoing PCR reaction are electrokinetically injected, separated by size via CE, and measured. By collecting several data points during the thermocycling process for each individual band, an amplification curve for each target is recorded, followed by automatic determination of its quantification cycle (Cq). This process enables high quality applications in diagnostics and life sciences that require the analysis of a medium-to-high number of molecular markers in parallel within a few hours.

The presented study shows the successful merging of two commercially available assays for infectious disease into one MODAPLEX multiplex panel, covering 10 diagnostic markers plus controls. This enables the simultaneous detection of several viral pathogens in a clinical setting, using a syndromic testing approach.

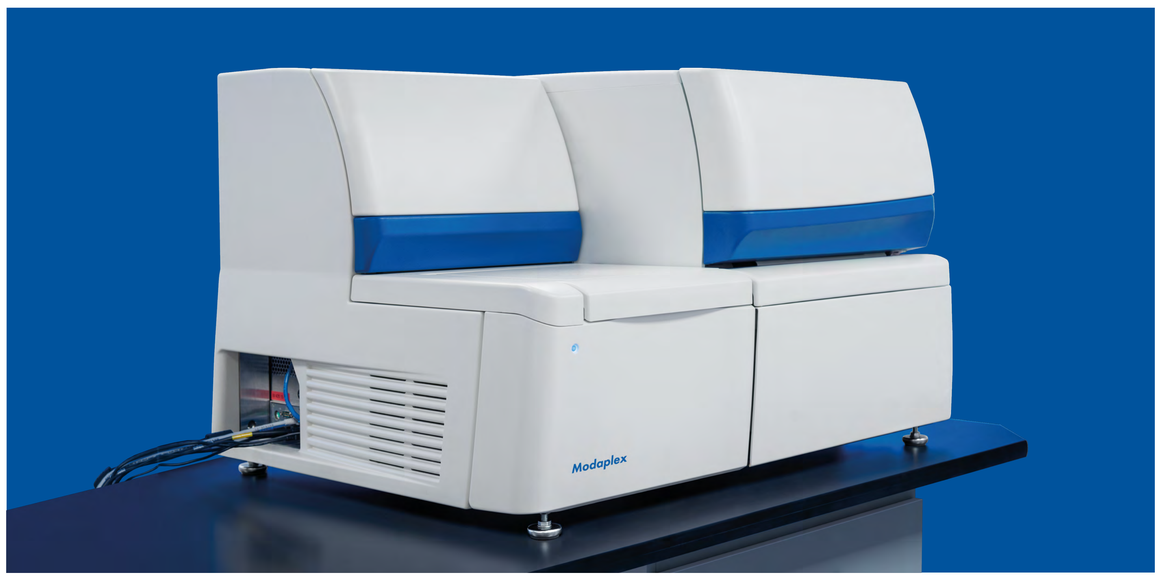

Figure 1: MODAPLEX platform

Methods

The combination of PCR and automated sequential CE was performed using a benchtop molecular genetic system (MODAPLEX, BIOTYPE GmbH, Dresden, Germany). After each PCR cycle, the fluorescently labeled fragments are electrokinetically injected into the capillary gel. While the PCR reaction continues undisturbed, the injected probe hydrolysis products are size separated from each other in the gel and detected by fluorescence. The synchronized PCR reaction and CE separations generate real-time data that allows quantification of the molecular targets.

For PCR setup the components (Mastermix, Positive control, Internal control) of the provided IVD kits were used. The composition (sequences and concentrations) of the primer/probe mix was disclosed by the manufacturer. All hydrolysis probes had to be modified on their 5'end by change of the fluorophores and adding CE-migration modifiers to enable the transfer on the MODAPLEX platform while keeping the sequences constant. The 3' end of each probe was blocked to avoid extension. The oligos of all four qPCR multiplexes were mixed in their original concentrations and not further optimized. Each PCR reaction contains, additionally to the assay-specific oligos, three non-amplifying size standards (SST) and a template independent amplification control (AC).

The hydrolysis fragments were separated based on their physical properties such as charge, hydrophobicity, size, and mass. The specificity of the hydrolysis was ensured by protecting distinct positions of each probe from the nuclease domain of the Taq Polymerase (see figure 2).

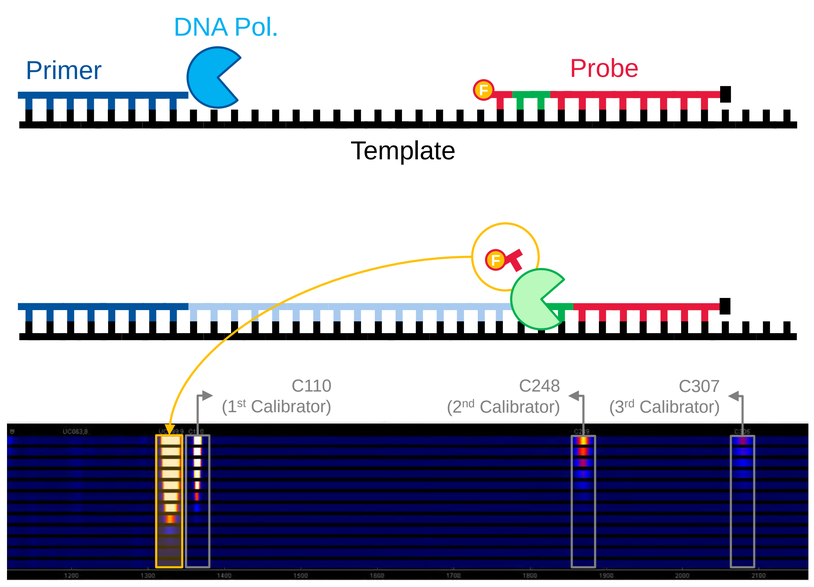

Figure 2: Schematic overview of the MODAPLEX technology for the application of hydrolysis probes on a CE based system. A locus or allele-specific primer (blue) initiates the DNA synthesis by a Taq DNA polymerase (light-blue) followed by hydrolysis of a locus or allele-specific probe (red). The probe contains a 5' fluorescent label, a 3' blocker to avoid extension and internal blocker (green) to define the hydrolysis site. The hydrolysed, fluorescent labelled 5' fragment of the probe (yellow) migrates with a characteristic migration length on the CE. This allows the direct transfer of hydrolysis probe based assays to a CE-based system (e.g. MODAPLEX) with a multiplex degree of up to 30.

The limit of detection (LOD) for all targets (SARS-CoV2, FluA, FluB, RSV, Mutations) was determined using synthetic RNA from Twist Bioscience (South San Francisco, CA, US) which was spiked into negative saliva samples and purified. The limit of blank (LOB) was determined with purified negative saliva samples. The validation was performed with pre-characterized clinical samples.

Results and Discussion

The detection of hydrolysis products on the MODAPLEX platform is not dependent on dequenching of different fluorophores as it is used for many different qPCR multiplex technologies, such as TaqMan™ or Molecular Beacons. Instead, the multiplex degree is achieved by separating the labeled hydrolysis products by CE and detecting their fluorescence (s. figure 2). Each fragment is labeled with a unique combination of fluorophore, linker and spacer, enabling parallel differentiation of up to 30 probes.

First, each singleplex and the existing multiplexes were tested to prove the transferability from a standard qPCR platform to the MODAPLEX device. After no problems were detected, all oligos were combined in one reaction and tested in parallel with the positive control (s. figure 3). All 11 targets (FluA, FluB, RSV, the SARS-CoV2 targets M-gene, S-gene, ORF1ab, and the SNPs of the S-gene K417T, K417N, E484K, and P681R) were reliably detected.

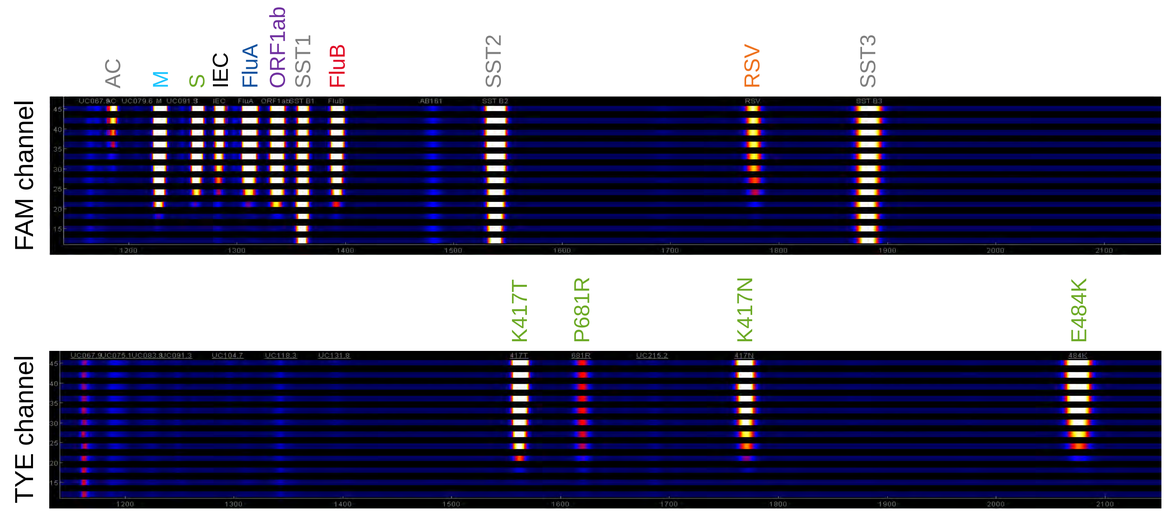

Figure 3: Gel views of both channels of MODAPLEX run for the positive control of the consolidated multiplex assay. The intensity of all target peaks increases over 12 CE separations, which are automatically taken during a thermocycling profile. AC, SST1, SST2, SST3: size standards and template independent PCR control. IEC: Internal extraction control. K417T, P681R, K417N and E484K: mutations of the S-protein of SARS-CoV2.

In July 2021, the National Health Service established a process (Coronavirus Test Device Approvals (CTDA)) in the UK to ensure quality standards of Coronavirus tests [2]. The minimal required LOD is specified as 1000 copies/ml sample, which corresponds to 5 copies per PCR reaction. Therefore, the LOD was not determined in detail for each target but proven for this benchmark. In addition to FluA, FluB and RSV, we tested the SARS-CoV2 variants Beta, Gamma, Kappa, and Omicron to cover all targets of the multiplex assay. For all targets, the required sensitivity of 5 copies per reaction was achieved and the performance reported in the IFUs was confirmed (see table 1). No unspecific amplification was observed. The immune escape mutations could even be detected with a higher analytical sensitivity than reported. The SARS-CoV2 variants were correctly identified down to 10 copies/reaction (see table 2).

Table 1: Comparison of reported LoD of the original multiplex assays (qPCR) with the consolidated assay on the MODAPLEX platform (N = 40 to 160).

	Reported LOD (qPCR) [copies]	MODAPLEX [copies]
ORF1ab	5	5
S	5	5
M	6	5
FluA	3	5
FluB	2	5
RSV	3	5
E484K	25	5
K417N	25	10
K417T	25	5
P681R	25	10

Table 2: LoD for determination of the SARS-CoV2 strain on the MODAPLEX platform (N = 40). Identifying mutations in brackets.

SARS-CoV2 strain	MODAPLEX [copies]
Beta	10 (K417N/E484K)
Gamma	10 (K417T/E484K)
Kappa	10 (P681R)
Omicron	20 (K417N)

After confirmation of the required analytical sensitivity, a small set of clinical samples (3 samples each) was tested. All samples were correctly identified with only minor variance between replicates (<3%CV, N=4). This means 100 % sensitivity and 100 % specificity for this preliminary data set (see figure 4).

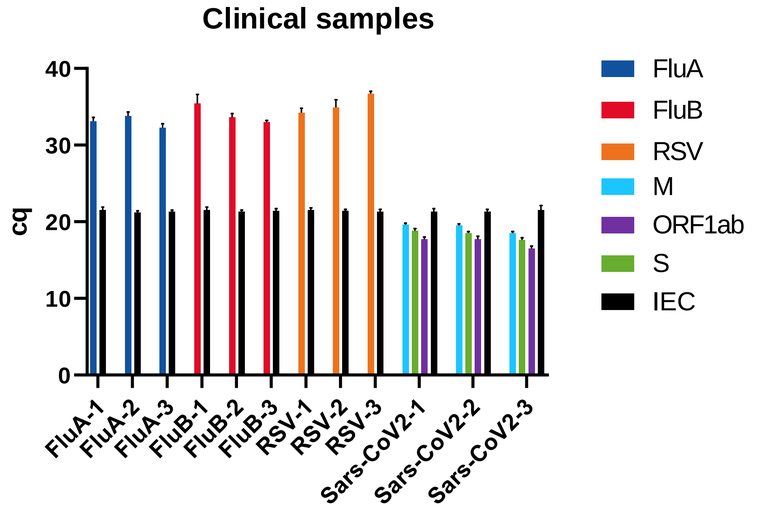

Figure 4: Results of the validation of the consolidated assay on the MODAPLEX with characterized clinical samples (N = 3). All samples were correctly identified. IEC: internal extraction control.

Conclusion

Two CE-IVD kits, each consisting of 2 multiplex qPCR assays, were merged on the MODAPLEX platform into an 11-plex, single-well assay with sustained performance. The LOD, the LOB and the clinical sensitivity, although tested on a limited data set, were confirmed, or exceeded. In conclusion, the MODAPLEX platform in combination with BIOTYPE's proprietary technology for hydrolysis probes and the established oligo design workflow, enables seamless and efficient consolidation of existing qPCR assays. Subsequently, the MODAPLEX technology reduces necessary PCR reactions, allowing high throughput, reduced hands-on-time and more comprehensive datasets. In summary, the MODAPLEX platform is a novel solution for syndromic testing in the clinical routine.

References

[1] Amy S Fox, Sonia N Rao, Syndromic testing for the diagnosis of infectious diseases: the right test if used for the right patient, Journal of Antimicrobial Chemotherapy, Volume 76, Issue Supplement_3, September 2021, Pages iii2–iii3,
[2] Hlousek L, et al. Automated high multiplex qPCR platform for simultaneous detection and quantification of multiple nucleic acid targets. Biotechniques. 2012 May;52(5):316-24.
[3] https://www.gov.uk/government/publications/assessment-and-procurement-of-coronavirus-covid-19-tests

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