SUPPLEMENTARY MATERIAL

Detection of SARS-CoV-2 virus by Triplex Enhanced Nucleic Acid Detection Assay (TENADA)

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INDEX

Table S1. Results of TFO target sequences search in the SARS-CoV-2 genome.

Sequences (5'-3')	Length	%GC	Orientation	Position
AGATGAGGATGAAGAAGAAGGTGA	24	41.7	Forward	3031
GAGCAGAAGGGTAGTAGAGAG	21	47.6	Reverse	17128
GTGATGAGGAACGAGAAGAGG	21	47.6	Reverse	28823
GAGGGAAGGACATAAGATGA	20	40.0	Reverse	2470

Table S2. Sequences of the PPRH and reporter probes (RP) used in this study.

Oligonucleotide	Sequence (5'-3')
PPRH-CC1	GAGCAGAAGGGTAGTAGAGAGTTTTGAGAGATGATGGGAAGACGAG
PPRH-CC2	GTGATGAGGAACGAGAAGAGGTTTTGGAGAAGAGCAAGGAGTAGTG
PPRH-CC3	GAGGGAAGGACATAAGATGATTTTAGTAGAATACAGGAAGGGAG
CC1-RP	GGCCAATAGCAAAATGACTC
CC2-RP	TGCGTAGAAGCCTTTTGGC
CC3-RP	CCCTTTCCACAAAAATCAAC

Table S3. Sequences of the oligonucleotides prepared in this work.

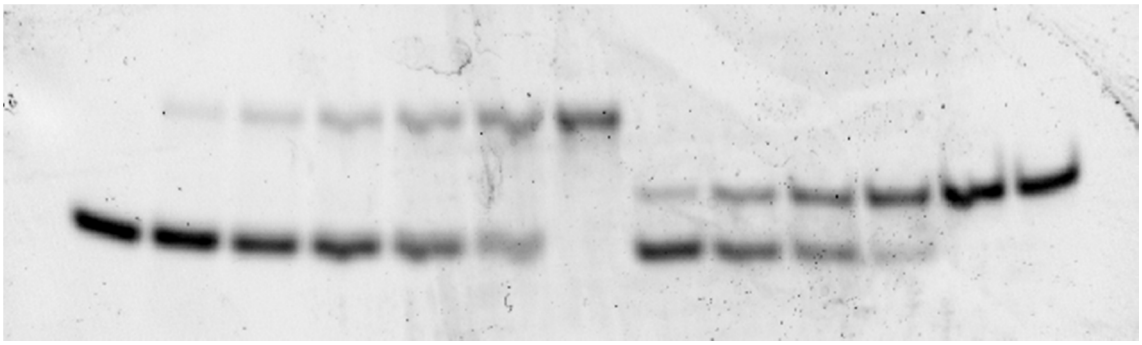
CC1 SYSTEM:
CC1PPRH-amino
5'-NH_2-TTTTTGAGCAGAAGGGTAGTAGAGAGTTTTGAGAGATGATGGGAAGACGAG-3'
CC1PPRH
5'-GAGCAGAAGGGTAGTAGAGAGTTTTGAGAGATGATGGGAAGACGAG -3'
CC1PPRH-Control (reverse Hoogsteen strand scrambled)
5'-GAGCAGAAGGGTAGTAGAGAGTTTTGGAGAGCAGGAATAGAGGAGT-3'
CC1duplex-amino
5'-NH_2-TTTTTGAGCAGAAGGGTAGTAGAGAG-3'
CC1biotineRP:
5'-GGCCAATAGCAAAATGACTC-BIOTINE-3'
CC1-TamraRP:
5'-GGCCAATAGCAAAATGACTC-Tamra-3'
CC1-Cy3RP:
5'-GGCCAATAGCAAAATGACTC-Cy3-3'
CC1-ThiolRP:
5'-GGCCAATAGCAAAATGACTC-Thiol-3'
CC1target:
5'-GAGTCATTTTGCTATTGGCCTAGCTCTCTACTACCCTTCTGCTC-3'
CC1DNAtarget-FAM
5'-FAM-GCTATTGGCCTAGCTCTCTACTACCCTTCTGCTCGCATAGTGTATAC-3'
CC1RNAtarget-FAM
5'-FAM-CUAGCUCUCUACUACCCUUCUGCUCGCAUA-3'

CC1DNAtargetLarge-FAM
5' FAM-GGTAAGAGTCATTTTGCTATTGGCCTAGCTCTCTACTACCCTTCTGCTCGCATA 3’

CC2 SYSTEM
CC2PPRH-amino:
5’-NH_2-TTTTTGTGATGAGGAACGAGAAGAGGTTTTGGAGAAGAGCAAGGAGTAGTG-3’
CC2PPRH:
5’-GTGATGAGGAACGAGAAGAGGTTTTGGAGAAGAGCAAGGAGTAGTG-3’
CC2duplex-amino:
5’-NH_2-TTTTTGTGATGAGGAACGAGAAGAGG-3’
CC2-biotineRP:
5’- TGCGTAGAAGCCTTTTGGC-biotine-3’
CC2-Cy3RP:
5’- TGCGTAGAAGCCTTTTGGC-Cy3-3’
CC2-ThiolRP:
5’- TGCGTAGAAGCCTTTTGGC-thiol-3’
CC2-biotineRPNew:
5’-CCTTCTGCGTAGAAGCCTTT -biotine-3’
CC2DNATarget:
5’-GCCAAAAGGCTTCTACGCAGAAGGGAGCAGAGGCGGCAGTCAAGCCTCTTCTCGTTCCTCATCAC-3’
CC2NewTarget:
5’-AAAGGCTTCTACGCAGAAGGGAGCAGAGGCGGCAGTCAAGCCTCTTCTCGTTCCTCATCAC-3’
CC2DNAtarget-FAM
FAM-5’- TCAAGCCTCTTCTCGTTCCTCATCACGTAGT-3’

CC3 SYSTEM
CC3PPRH-amino:
5’-NH_2-TTTTTGAGGGAAGGACATAAGATGATTTTAGTAGAATACAGGAAGGGAG-3’
CC3PPRH:
5’-GAGGGAAGGACATAAGATGATTTTAGTAGAATACAGGAAGGGAG-3’
CC3duplex-amino:
5’-NH_2-TTTTTGAGGGAAGGACATAAGATGA-3’
CC3-biotineRPnew1:
5’-CCCTTTCCACAAAAATCAAC-BIOTINE-3’
CC3-biotineRP:
5’- CCCTTTCCACAAAAATCAAC-biotine-3’
CC3-Cy3RP:
5’- CCCTTTCCACAAAAATCAAC-Cy3-3’
CC3-thiolRP:
5’- CCCTTTCCACAAAAATCAAC-thiol-3’
CC3DNAtarget:
5’-GTTGATTTTTGTGGAAAGGGCTATCATCTTATGTCCTTCCCTC-3’
CC3DNAtarget-FAM
FAM-5’-GTGGAAAGGGCTATCATCTTATGTCCTTCCCTCAGTCAGCACCTCAT-3’
CC3RNAtarget-FAM
FAM-5’-GGCUAUCAUCUUAUGUCCUUCCCUCAGUCA-3’

Figure S1. Binding of CC2-DNA-target with NH_2-PPRH-CC2and NH_2-CC2duplex



K_d PPRH = $6.04 \cdot 10^{-7}$ M K_d duplex = $7.47 \cdot 10^{-7}$ M

Table S4. Dissociation constants of PPRH-CC1, CC2, CC3 and PPRH-CC1-control and duplex-CC1, CC2 and CC3.

Oligonucleotide	K_d (10^{-7}), M
PPRH-CC1[#]	3.88
Duplex-CC1[#]	4.79
PPRH-CC2[&]	6.04
Duplex-CC2[&]	7.47
PPRH-CC3[*]	3.86
Duplex-CC3[*]	10.5
PPRH-CC1Control[#]	6.24

[#]Target oligonucleotide used: CC1DNAtargetLarge-FAM;
[&]Target oligonucleotide used: CC2DNAtarget-FAM;
[*]Target oligonucleotide used: CC3DNAtarget-FAM;

Table S5. Limit of detection values (LoD, expressed in nanomolar, nM) corresponding to the CC pairs calibration curves obtained in a multiplex assay. The table shows the data obtained when diluting the corresponding targets in different Universal Transport media (UTMs 1-3) compared with the values achieved when using hybridization buffer. In this case, the first oligonucleotides (PPRH and duplex format) were printed on the glass slide at a concentration of 125 nM. Subsequently, serial target dilutions were added (ranging from 500 nM to 0 with a dilution factor of 5). Finally, the labelled second oligonucleotides were added at an optimized concentration of 250 nM.

Buffer/UTM	CC1		CC2		CC3	
	PPRH	Duplex	PPRH	Duplex	PPRH	Duplex
Hybridization buffer	1.01	0.64	3.16	4.67	1.56	3.49
UTM 1	0.47	0.61	2.53	2.60	2.58	3.76
UTM 2	0.01	0.06	2.03	1.23	0.72	0.33
UTM 3	0.03	0.02	0.89	0.71	1.50	1.17