Performance Evaluation Summary of Fluxergy Test Kit COVID-19

(BS EN 13612:2002 – Performance evaluation of in vitro diagnostic medical devices)

1. Product Name

Fluxergy Test Kit COVID-19

2. Intended Use

Fluxergy Test Kit COVID-19 is a real-time reverse transcriptase (RT) polymerase chain reaction (PCR) test intended for the qualitative detection of nucleic acid from SARS-CoV-2 in nasopharyngeal swabs (NPS) collected by a healthcare worker from patients suspected of COVID-19 by their healthcare provider. Testing should be performed by laboratories or in other qualified professional care settings that meet their local registration or licensing requirements for in-vitro diagnostic testing.

Results are for the identification of SARS-CoV-2 RNA. The SARS-CoV-2 RNA is generally detectable in respiratory specimens during the acute phase of infection. Positive results are indicative of the presence of SARS-CoV-2 RNA; clinical correlation with patient history and other diagnostic information is necessary to determine patient infection status. Positive results do not rule out bacterial infection or co-infection with other viruses.

Negative results do not preclude SARS-CoV-2 infection and should not be used as the sole basis for patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information.

The Fluxergy Test Kit COVID-19 is intended for use by qualified and trained clinical laboratory personnel specifically instructed and trained in the techniques of real-time PCR and in vitro diagnostic procedures.

3. Explanation of the Test

The Fluxergy Test Kit COVID-19 is a qualitative real-time reverse transcription polymerase chain reaction (RT-qPCR) test. The SARS-CoV-2 primer and probe set is designed to detect RNA from SARS-CoV-2 in nasopharyngeal swabs (NPS) from patients with signs and symptoms of infection who are suspected of COVID-19. The test performance is monitored by standardized internal controls and provides results within 1 hour from when the test is initiated with a suspected sample.

4. Principles of the Procedure

The Fluxergy Test Kit COVID-19 enables processing, amplification, and detection of SARS-CoV-2 RNA from infected nasopharyngeal swabs samples. The assay consists of one Fluxergy Reaction Mix COVID-19 and one Fluxergy Card. The assay is performed on the Fluxergy Analyzer instrument which is controlled by an external computer equipped with Fluxergy Works Software.

The Fluxergy Card is a disposable card into which the PCR reagents mixed with test samples are manually pipetted in. Each card contains a single sample/reagent input well and microfluidic channels that control the flow of liquid, and reaction wells. The Fluxergy Card is self-contained to prevent cross-contamination between samples.

In the Fluxergy Test Kit COVID-19, fluorescent probes are used together with corresponding forward and reverse primers to amplify SARS-CoV-2 RNA and exogenous internal control. Two well-conserved regions of the SARS-CoV-2 genome are targeted to identify SARS-CoV-2 RNA in the specimen. Internal control is used to detect PCR failure and/or inhibition in addition to monitoring adequate sample processing.

The Fluxergy Analyzer instrument is a rapid qPCR thermocycler used for the identification of nucleic acid from biological specimens. The Fluxergy Analyzer performs amplification, detection, and analysis of fluorescent signals generated during PCR.

The NPS sample collected in viral transport media is mixed with ready-to-use Fluxergy Reaction Mix COVID-19 to prepare the complete test master mix. The master mix is then loaded onto the Fluxergy Card COVID-19. After loading the Fluxergy Card into the Fluxergy Analyzer instrument, the run is initiated. Approximately in 1 hour, Fluxergy Works will complete the thermal cycling and analysis.

5. Performance Evaluation

5.1 Clinical Evaluation

The clinical performance evaluation of the Fluxergy Test Kit COVID-19 was conducted with archived clinical nasopharyngeal swabs (NPS) in viral transport medium. A total of 95 NPS samples (45 SARS-CoV-2 positive and 50 SARS-CoV-2 negative) were collected from November 2020 to March 2021 during the COVID-19 pandemic in the United States. All samples had been confirmed as positive or negative for SARS-CoV-2 by an authorized RT-PCR test (WHO EUL, US FDA EUA, CE-IVD, Health Canada, Australia TGA, etc.)

The randomized and blinded samples were tested with the Fluxergy Test Kit COVID-19 using Fluxergy Analyzer to generate the Positive Percent Agreement (PPA) and Negative Percent Agreement (NPA) as an estimate for diagnostic accuracy (sensitivity/specificity). PPA and NPA were determined by comparing results of the Fluxergy Test Kit COVID-19 against the expected results. Results of these 95 archived clinical NPS samples are shown in table below.

Clinical Performance Results of Fluxergy Test Kit COVID-19			
	Expected Results		
Fluxergy Test Kit COVID-19	**Positive**	**Negative**	**TOTAL**
Positive	45	0	***45***
Negative	0	50	***50***
TOTAL	***45***	***50***	***95***
PPA	***100.00%*** *(95% CI: 92.31% - 100.00%)**		
NPA	***100.00%*** *(95% CI: 92.89% - 100.00%)**		

* *The 95 samples required 108 test runs to obtain the results. Error codes required 13 retests of 12 samples to confirm final results.*

5.2 Analytical Evaluation

1. Limit of Detection (LoD) – Analytical Sensitivity

The LoD of the Fluxergy Test Kit COVID-19 was determined using heat inactivated particles of SARS-Related Coronavirus 2 (SARS-CoV-2, Isolate: USA-WA1/2020). This virus was originally isolated from a patient with COVID-19 (GenBank MN985325). The cultivated virus titered by an infectious test and subsequently heat inactivated by the vendor (Zeptometrix Catalog # 0810587CFHI-0.5mL). Viral inactivation was verified by the absence of viral growth in tissue culture-based infectivity assays.

A preliminary LoD was determined by testing in triplicate 10-fold serial dilutions of the heat inactivated virus spiked into pooled negative nasopharyngeal swab (NPS) matrix. NPS samples were collected in Puritan UniTranz-RT 3mL Universal Transport Solution (SKU#: UT-300). The collected NPS matrix samples were qualified as negatives by RT-PCR (Fluxergy's COVID-19 TaqMan assay) before using them for virus spiking. The tentative LoD was confirmed by testing an additional 20 replicates. The limit of detection was defined as the lowest concentration at which each target is detected at least 95% of the time. The claimed LoD of the Fluxergy Test Kit COVID-19 is 0.3 $TCID_{50}$/mL.

LoD Determination using USA-WA1/2020 Strain	
Concentration Tested	**# Tested / # Detected (%)**
0.3 $TCID_{50}$ / mL.	20/20 (100%)

2. Inclusivity – Analytical Reactivity

The inclusivity of the Fluxergy's Test Kit COVID-19 was evaluated using *in-silico* analysis of the assay primers and probes against 54,652 sequences available in the Global Initiative on Sharing Avian Influenza Data (GISAID) and the National Center for Biotechnology Information (NCBI) databases for two gene targets, N and ORF1ab.

The analysis demonstrated that the regions recognized by the Fluxergy Test Kit COVID-19's primers and probes have >= 98.95% homology with all available SARS-CoV-2 sequences.

The nucleotide mismatches observed were predicted to have no impact on the assay performance. In addition, a two-target assay design mitigates the occurrence of false negative results due to failure to amplify the individual target sequences.

In-Silico Inclusivity Report				
	% Identity to N gene		% Identity to ORF1ab	
Database	Probe (% Match)	Primers (% Match)	Probe (% Match)	Primers (% Match)
NCBI	5049/5055* (99.88%)	5055/5055 (100%)	4497/4541* (99.03%)	4507/4541* (99.25%)
GISAID	49073/49597* (98.95%)	49482/49597* (99.77%)	49512/49597* (99.83%)	49535/49597* (99.87%)

* The nucleotide mismatches have no predicted impact on the assay performance.

3. Cross-Reactivity - Analytical Specificity (Exclusivity)

The Fluxergy Test Kit COVID-19 was tested for its specificity by both direct testing of organisms (wet-lab testing) and by *in-silico* analysis.

Wet Lab Testing

Cross-reactivity of Fluxergy Test Kit COVID-19 was evaluated by wet-lab testing using the Fluxergy Analyzer. A panel of 18 micro-organisms presented below, were tested at the indicated concentrations (8.9 x 10^5 TCID50 per mL to 2.8 x 10^8 CEID50 per mL). For some pathogens tested, titer was estimated based on their total nucleic acid concentration of stock material. These organisms are on the high priority list due to the reasonable likelihood they may be present in upper respiratory samples. The pathogen stocks were diluted in pooled NPS matrix (Nasopharyngeal swab samples were collected in Puritan UniTranz-RT 3mL Universal Transport Solution (SKU#: UT-300) and then qualified as negatives by RT-PCR with Fluxergy's COVID-19 TaqMan assay) to reduce the effect microbial growth media and to obtain the desired testing concentrations. 14 microliters of prepared pathogen stocks were then used to prepare master mix and set up cross-reactivity runs.

None of the organisms listed in below table interfered with Fluxergy Test Kit COVID-19 performance by generating false-positive results.

Cross-Reactivity of Fluxergy Test Kit COVID-19 (Wet Lab Testing)			
Organism	**Strain**	**Cat # (BEI Resources)**	**Concentration**
Bordetella holmesii	H785	NR-44175	2.04 x 10^8 copies/mL*
Candida albicans	23R	NR-29339	2.2 x 10^7 copies/mL*
Enterovirus D68	US/IL/14-18952	NR-49131	1.6 x 10^7 $TCID_{50}$/mL

Cross-Reactivity of Fluxergy Test Kit COVID-19 (Wet Lab Testing)			
Organism	**Strain**	**Cat # (BEI Resources)**	**Concentration**
Haemophilus haemolyticus	F0397	HM-469	7.5×10^7 /mL*
Human Coronavirus NL63	NL63	NR-470	5.5×10^5 $TCID_{50}$/mL
Human respiratory syncytial A	A2000/3-4	NR-28530	2.8×10^6 $TCID_{50}$/mL
Human respiratory syncytial B	B1	NR-4052	4.4×10^5 $TCID_{50}$/mL
Influenza A virus (H1N1)	A/Beijing/262/1995 (H1N1)	NR-12277	2.8×10^8 $CEID_{50}$/mL
Influenza A virus (H3N2)	A/Brisbane/10/2007 (H3N2)	NR-12283	2.2×10^8 $CEID_{50}$/mL
Influenza A Virus pdm09	A/NewYork/18/2009 (H1N1)pdm09	NR-49451	1.3×10^{10} copies/ mL*
Influenza B virus (Victoria)	B/Brisbane/60/2008	NR-42005	6.25×10^6 $CEID_{50}$/mL
MERS-Coronavirus	EMC/2012	NR-50549	8.9×10^5 $TCID_{50}$/mL
Pseudomonas aeruginosa	EnvKY1	NR-51329	1.4×10^7 copies/mL*
Rhinovirus 50	A2 #58	NR-51455	2×10^6 $TCID_{50}$/mL
SARS-CoV	Urbani strain	NR-9548	1×10^8 pfu/mL
Staphylococcus epidermidis	VCU013	NR-9548	8.5×10^5 copies/mL
Streptococcus pneumoniae	EMC23F	NR-51859	4.18×10^6 copies/mL*
Streptococcus pyogenes	ABC020063118	NR-48702	6.3×10^6 copies/mL*

* copies/mL, calculated based on the total nucleic acid concentration of extracted stock material.

In silico Analysis

To complete the analysis for cross-reactivity, the forward primer, reverse primer and probe sequences (both N and orf1ab) were blasted individually against the following 34 taxids. Results of the *in-silico* analysis are summarized in table below.

Cross-Reactivity of Fluxergy Test Kit COVID-19 (*in silico* analysis)		
Organism	**In-Silico Analysis for % Identity***	
	nCoV- N gene	**nCoV- orf1ab gene**
Adenoviridae (taxid:10508)	No significant similarity found	No significant similarity found
Human metapneumovirus (taxid:162145)	No significant similarity found	No significant similarity found
Human parainfluenza virus 1 (taxid:12730)	No significant similarity found	No significant similarity found
Human parainfluenza virus 2 (taxid:1979160)	No significant similarity found	No significant similarity found

Cross-Reactivity of Fluxergy Test Kit COVID-19 (*in silico* analysis)		
Organism	**In-Silico Analysis for % Identity***	
	nCoV- N gene	**nCoV- orf1ab gene**
Human parainfluenza virus 3 (taxid:11216)	No significant similarity found	No significant similarity found
Human parainfluenza virus 4 (taxid:1979161)	No significant similarity found	No significant similarity found
Chlamydia pneumoniae (taxid:83558)	No significant similarity found	No significant similarity found
Haemophilus (taxid:724)	No significant similarity found	No significant similarity found
Streptococcus pneumoniae (taxid:1313)	No significant similarity found	No significant similarity found
Streptococcus pyogenes (taxid:1314)	No significant similarity found	No significant similarity found
Bordetella pertussis (taxid:520)	No significant similarity found	No significant similarity found
Bordetella holmesii (taxid:35814)	No significant similarity found	No significant similarity found
Mycoplasma pneumoniae (taxid:2104)	No significant similarity found	No significant similarity found
Pneumocystis jirovecii (taxid:42068)	No significant similarity found	No significant similarity found
Parechovirus (taxid:138954)	No significant similarity found	No significant similarity found
Candida albicans (taxid:5476)	No significant similarity found	No significant similarity found
Corynebacterium diphtheriae (taxid:1717)	No significant similarity found	No significant similarity found
Legionella (taxid:445)	No significant similarity found	No significant similarity found
Bacillus anthracis (taxid:1392)	No significant similarity found	No significant similarity found
Moraxella catarrhalis (taxid:480)	No significant similarity found	No significant similarity found
Neisseria elongata (taxid:495)	No significant similarity found	No significant similarity found
Neisseria meningitidis (taxid:487)	No significant similarity found	No significant similarity found
Pseudomonas aeruginosa group (taxid:136841)	No significant similarity found	No significant similarity found
Staphylococcus epidermidis (taxid:1282)	No significant similarity found	No significant similarity found
Leptospiraceae (taxid:170)	No significant similarity found	No significant similarity found
Chlamydia psittaci (taxid:83554)	No significant similarity found	No significant similarity found
Coxiella burnetii (taxid:777)	No significant similarity found	No significant similarity found
Staphylococcus aureus (taxid:1280)	No significant similarity found	No significant similarity found
Adenoviridae (taxid:10508)	No significant similarity found	No significant similarity found
Mycobacterium tuberculosis (taxid:1773)	No significant similarity found	No significant similarity found
Streptococcus salivarius (taxid:1304)	No significant similarity found	No significant similarity found

Cross-Reactivity of Fluxergy Test Kit COVID-19 (*in silico* analysis)		
Organism	**In-Silico Analysis for % Identity***	
	nCoV- N gene	**nCoV- orf1ab gene**
Human coronavirus 229E (taxid:11137)	No significant similarity found	No significant similarity found
Human coronavirus OC43 (taxid:31631)	No significant similarity found	No significant similarity found
Human coronavirus HKU1 (taxid:290028)	No significant similarity found	No significant similarity found

* The amplicon sequences were blasted against low stringency filter [Somewhat similar sequences (blastn)].
No alignment showed >70% homology and hence no potential for cross-reactivity.

Based on the combined wet lab testing and in silico analysis, there is no potential unintended cross-reactivity with other organisms listed in both tables above.

4. Interference Study

Potential interfering substances from upper respiratory specimens were tested using samples containing the heat inactivated virus at 3x LoD in nuclease free water. Testing was performed with the Fluxergy Test Kit COVID-19 using the Fluxergy Analyzer and included triplicate testing per substance at the indicated levels.

Assay Interference Verification				
Potential Interfering Substance	**Active Ingredient**	**Conc Tested**	**SARS-CoV-2 Detection (#Detected / #Tested)**	**IC % Detection (#Detected / #Tested)**
Decongestant	Afrin Nasal Spray-Oxymetazoline	15% (v/v)	100% (3/ 3)	100% (3/ 3)
Antibacterial	Tobramycin	4 µg/mL	100% (3/ 3)	100% (3/ 3)
Antibiotic	Amoxicillin	0.5 mg/mL	100% (3/ 3)	100% (3/ 3)
Antibiotic	Cephalexin	0.04 mg/mL	100% (3/ 3)	100% (3/ 3)
Antibiotic	Clindamycin	0.03 mg/mL	100% (3/ 3)	100% (3/ 3)
Antibiotic	Erythromycin	1 mg/mL	100% (3/ 3)	100% (3/ 3)
Antibiotic	Mupirocin	6.6 mg/mL	100% (3/ 3)	100% (3/ 3)
Antibiotic	Penicillin	1200 U/mL	100% (3/ 3)	100% (3/ 3)
Antiviral Drug	Zanamivir	3.3 mg/mL	100% (3/ 3)	100% (3/ 3)
Aspirin	Aspirin	0.62 mg/mL	100% (3/ 3)	100% (3/ 3)
Benadryl	Diphenhydramine	10 µL/Rxn	100% (3/ 3)	100% (3/ 3)
Blood	N/A	2% (v/v)	100% (3/ 3)	100% (3/ 3)
Corticosterone	Corticosterone	4 mg/swab	100% (3/ 3)	100% (3/ 3)
Corticosterone	Fluticasone	5% (v/v)	100% (3/ 3)	100% (3/ 3)
Mucin Protein	Bovine	60 µg/mL	100% (3/ 3)	100% (3/ 3)
Neo-Synephrine	Phenylephrine HCl	0.16 mg/mL	100% (3/ 3)	100% (3/ 3)
Nyquil	Dextromethorphan; Hydrobromide; Doxylamine Succinate	1/200 Dilution	100% (3/ 3)	100% (3/ 3)
Pain Medication	Acetaminophen	1 mg/mL	100% (3/ 3)	100% (3/ 3)

Assay Interference Verification				
Potential Interfering Substance	**Active Ingredient**	**Conc Tested**	**SARS-CoV-2 Detection (#Detected / #Tested)**	**IC % Detection (#Detected / #Tested)**
Pain Medication	Nonsteroidal anti-inflammatory drug	1 mg/mL	100% (3/ 3)	100% (3/ 3)
Robitussin Cough	Dextromethorphan HBr; Guaifenesin	2.0 mg/mL	100% (3/ 3)	100% (3/ 3)
Tamiflu Antiviral Drug	Oseltamivir	1 µM	100% (3/ 3)	100% (3/ 3)
Nasal Spray	Saline	15% (v/v)	100% (3/ 3)	100% (3/ 3)
Sore Throat Lozenge	Menthol	1 mg/mL	100% (3/ 3)	100% (3/ 3)
Sore Throat Lozenge	Zinc Gluconate Glycine	1 mg/mL	100% (3/ 3)	100% (3/ 3)
Zicam Nasal Gel	Oxymetazoline hydrochloride	5% (v/v)	100% (3/ 3)	100% (3/ 3)
NA	Non-spike Control	3x LoD	100% (3/ 3)	100% (3/ 3)

5. Reproducibility Study

Inter-assay precision was tested to assess the reproducibility of results. Three samples (1.5x LoD, 3x LoD and 5x LoD, prepared by spiking heat-inactivated SARS-CoV-2 virus) were tested in 4 replicates using 2 lots of Fluxergy Kit lots over 5 days. The study generated a total of 40 replicates per test sample (5 days x 4 replicates x 2 kit lots = 40 replicates per test level). The variance component analysis performed showed the standard deviation and %CV of Ct values between lots & days to be <1.5 and <4.5 %, respectively.

Precision test results between Lots (SARS-CoV-2 Results)				
Sample	**KIT Lot #**	**Mean Ct**	**Std Dev (Ct)**	**% CV (Ct)**
Zepto 1.5x LoD	1	35.36	0.98	2.76
Zepto 1.5x LoD	2	34.72	0.65	1.86
Zepto 3x LoD	1	34.44	0.84	2.44
Zepto 3x LoD	2	34.54	1.11	3.22
Zepto 5x LoD	1	34.08	1.42	4.16
Zepto 5x LoD	2	34.02	0.63	1.85

Precision test results between Days (SARS-CoV-2 Results)				
Sample	**Day**	**Meant Ct**	**Std Dev (Ct)**	**% CV (Ct)**
Zepto 1.5x LoD	1	35.39	0.94	2.65
Zepto 1.5x LoD	2	35.09	0.98	2.79
Zepto 1.5x LoD	3	35.29	0.55	1.57
Zepto 1.5x LoD	4	35.20	0.94	2.68

Zepto 1.5x LoD	5	34.24	0.53	1.54
Zepto 3x LoD	1	34.94	1.01	2.89
Zepto 3x LoD	2	34.56	0.83	2.40
Zepto 3x LoD	3	34.22	1.33	3.89
Zepto 3x LoD	4	34.46	0.72	2.08
Zepto 3x LoD	5	34.28	0.96	2.79
Zepto 5x LoD	1	34.15	0.77	2.27
Zepto 5x LoD	2	34.16	1.17	3.42
Zepto 5x LoD	3	33.33	1.04	3.13
Zepto 5x LoD	4	34.29	0.87	2.54
Zepto 5x LoD	5	34.34	1.40	4.07

The validation study passed as the %CV was <10%

6. Revision History

Version	Date	Reason for Change
1	May 3, 2021	Initial Revision