New Molecular Screening Assay for Increased Detection Rate of *CFTR* Mutations in European Populations

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Abstract

The xTAG® Cystic Fibrosis 71 v2 kit (Luminex 200) is a new semi-quantitative, fully automated molecular genotyping test developed to simultaneously detect and identify an enlarged number of mutations and variants in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. During a validation process, the Luminex method was compared with INNO-LiPA® (Innogenetics), used as a reference method. A total of 120 DNA samples extracted from different biological matrices, including blood samples, blood spots from Guthrie cards, chorionic villi and amniotic fluid, were tested. Reproducible and concordant results were obtained on the Luminex platform from all DNA Innogenetics positive samples. However, differences were observed in the sensitivity of the methods based on distinct compositions of mutation panels. Indeed, 43 additional *CFTR* changes beyond those present in the Innogenetics panel are tested in the Luminex platform; of those, 40 are known mutations and three are recommended interference benign variants of F508del (F508C, I507V and I506V). Extension of the mutation panel to include Q552X, 711+5G>A, S1251N and nine other mutations is currently undergoing a validation process and will place Luminex xTAG as the most sensitive platform for routine *CFTR* molecular diagnosis in European countries.

Keywords

Cystic fibrosis, cystic fibrosis transmembrane conductance regulator (CFTR), screening, molecular diagnosis

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Cystic fibrosis (CF) is the most common and lethal hereditary recessive disease in Caucasian populations. The disease is caused by mutations of the CF transmembrane conductance regulator (*CFTR*) gene, located on the 7q31.2 locus, whose predicted translation product is a 1,480 amino acid protein. The protein, expressed in a variety of target tissues, organs and exocrine glands, functions as a cyclic adenosine monophosphate (cAMP)-dependent low-conductance chloride channel. The disease is less common in Hispanics and African-Americans[1] and is relatively rare in Asian Americans, with an incidence of one in 32,100. In central Europeans its frequency reaches about one in 2,500.

To date, over 1,600 different mutations have been identified (www.genet.sickkids.on.ca/). The most common mutation worldwide results in a deletion of a single phenylalanine residue at position 508 (F508del) and causes defective synthesis and folding of the mutant protein, which fails to escape the endoplasmic reticulum and reach the apical membrane of many epithelial cells.[2] Apart from the F508del mutation, which is responsible for approximately two-thirds of all CF chromosomes with a clear north-west–south-west gradient in its frequency across Europe,[3] there is a core of 38 less common mutations that occur with a relative frequency in European countries of 0.1% or greater.[4] The remainder, considered as rare mutations, have been identified in few individuals. Ethnic and racial backgrounds influence both the nature and the distribution of *CFTR* mutations. CF disease has a complex phenotype with variable disease severity and multiple clinical manifestations, including sino-pulmonary disease, exocrine pancreatic insufficiency, male infertility and high concentrations of sweat electrolytes. Patients displaying classic characteristics of CF from infancy usually have a relatively poor prognosis. There has been growing recognition of atypical, milder, pauci-organ disease cases of CF presenting in adolescence or adulthood and displaying normal or borderline sweat test and a better prognosis for survival.[5] Additionally, growing evidence of an association between *CFTR* mutations and other diseases, including isolated idiopathic pancreatitis, chronic rhinosinusitis, nasal polyposis, idiopathic bronchiectasis, allergic bronchoalveolar aspergillosis and congenital absence of vas deferens, has been added to this complex picture.[5] Atypical CF and CF-related disorders have made the diagnosis of CF less straightforward for clinicians and for geneticists, and have also required further extensive genetic screening.

Multiple *CFTR* molecular assays using diverse technologies are commercially available for routine analyses. Screening panels of *CFTR* mutations have been designed based on selected mutations having higher frequency among North Caucasian populations. Validation of a

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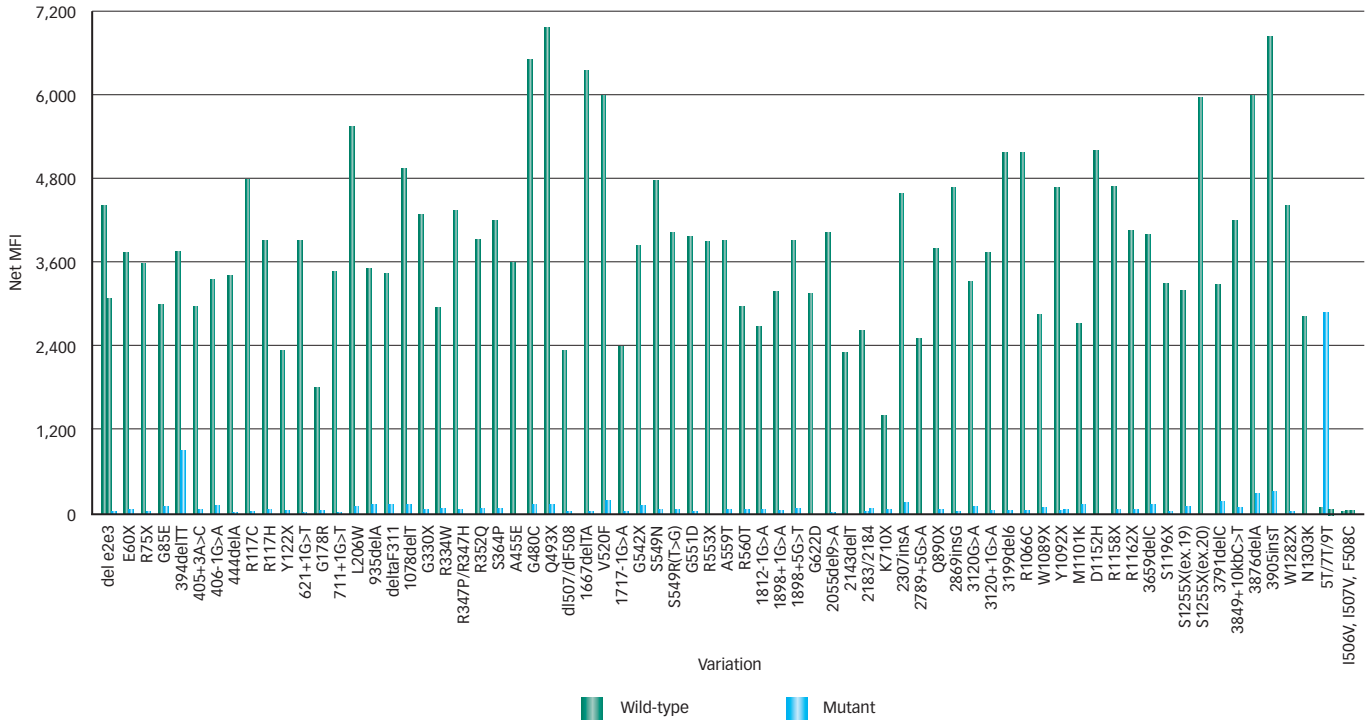
Figure 1: Histogram of Wild-type and Mutant Net Signals of Variants Obtained from an Individual Wild-type *CFTR* DNA Sample



Expressed as median fluorescence intensity (MFI). The polythymidine tract of intron 8 shows a homozygous 7T/7T genotype.

CFTR molecular test should integrate distinct analytical steps encompassing sensitivity, specificity, accuracy, signal discrimination and no call signal or repeat rate. Practical aspects comprising integration of the required platform into laboratory workflow, data interpretation methods and required software, hands-on or start-to-finish time and cost analyses should also be taken into account. The mutation panel and its sensitivity to the target populations are pivotal decisional factors. Recommendations of the American College of Medical Genetics (ACMG) include a minimal list of 23 *CFTR* mutations as well as reflex testing for R117H of the Tn polymorphic alleles in intron 8 and interference testing for benign F508del variants such as F508C, I507V and I506V.[6]

Over 10 years of experience with *CFTR* molecular tests, our laboratory has used screening platforms allowing continuously upgrading mutation panels, from 13 in 1999 to 20 up to 2002 (ARMS, Elucigene CF), then to 29 up to 2004 and finally to 36 until 2008 (INNO-LiPACFTR17+TnUpdate and INNO-LiPACFTR19 kit; AUTO-LiPA instrument, Innogenetics, Ghent, Belgium). More recently, we have been involved in the pilot validation process of a new molecular screening method, the xTAG® Cystic Fibrosis 71 v2 kit (Luminex 200 Instrument; Luminex Co, Austin, Texas, US). During the validation process, the Innogenetics method was used as a reference method.

Luminex xTAG is a semi-quantitative, fully automated genotyping test developed to simultaneously detect and identify an enlarged number of mutations and variants in the *CFTR* gene. The Luminex platform is a fast, easy, robust and highly specific DNA hybridisation test incorporating multiplex polymerase chain reaction (PCR) and multiplex allele-specific primer extension with Luminex tagged primers, which are composed of an inventive array of fluorescently labelled microsphere beads. Signals generated for each specific mutation and variant are analysed on the proprietary platform and are finally expressed as net values, allelic ratios and thresholds, which are calculated by comparison with those signals generated for the corresponding wild-type alleles. Output files are then generated and a four-page report including a one-page colour histogram can be printed out for each individual mutation and assay run. A colour histogram obtained from an individual wild-type *CFTR* DNA sample is depicted in *Figure 1*. An example of a heterozygous F508del DNA sample is shown in *Figure 2*.

Internal Validation Process of the Luminex Method

During an internal validation process, a total of 120 DNA samples extracted from different biological matrices, including blood samples, blood spots obtained from Guthrie cards, chorionic villi and amniotic fluid, were tested by both the new and the reference method. Each sample was analysed in triplicate, in three different runs performed on different days by three different operators. Data analyses were carried out by applying working protocols according to the corresponding manufacturer's recommendations. Data interpretation of Luminex test products was performed using Luminex software. Costs as well as hands-on time were similar for both methods. Start-to-finish time for a run of 45 DNA samples on the Luminex platform averaged one day of a technician's time. No significant inter-operator variability was noted. No call rate was acceptable (<1%). Reproducible and concordant results were obtained on the Luminex platform from all DNA Innogenetics positive samples. A comparison between methods obtained for the length of the intron 8 polytymidine tract, tested in both panels, showed that they were identical. However, differences were observed in the sensitivity of the methods based on the distinct composition of mutation panels (see *Table 1*). Indeed, 43 additional *CFTR* changes, beyond those present in the Innogenetics panel, are tested in the Luminex platform; of these,

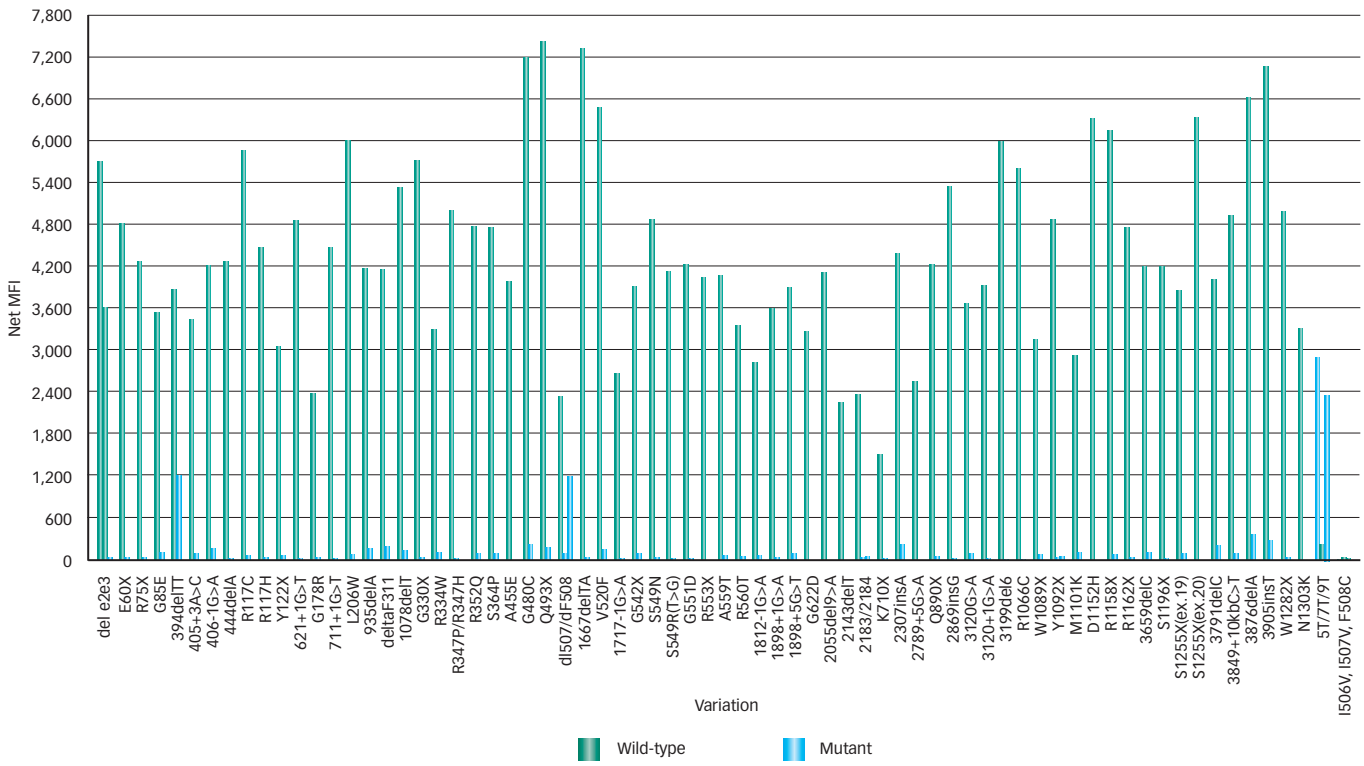
Table 1: Overview of Mutation Panels of Luminex xTAG Cystic Fibrosis 71 v2 and INNO-LiPA CFTR19 + CFTR17 +Tn Update, and Their Sensitivity in Different Selected Populations

				xTAG CF71v2 (Luminex)				
INNO-LiPA CFTR19 + CFTR17+Tn Update (Innogenetics)								
F508del	621+1G>T	R117H	3199del6	Y122X	M1101K	L206W	1812-1G>A	R1158X
1078delT	711+1G>T	R334W	CFTRdel2,3	R347H	S1255X	935delA	G622D	3791delC
1717-1G>A	A455E	R347P	3905insT	V520F	3876delA	F311del	2055del9>A	S1196X
1898+1G>A	I507del	R553X	394delTT	A559T	R75X	G330X	K710X	3120G>A
2184delA	G542X	R560T	5T/7T/9T	S549N	405+3A>C	R352Q	Q890X	F508C*
2789+5G>A	G551D	W1282X	Q552X**	S549R	406-1G>A	S364P	2869insG	I1507V*
3120+1G>A	G85E	E60X	711+5G>A**	1898+5G>T	444delA	G480C	R1066C	I506V*
3659delC	N1303K	2143delT	3272-26A>G**	2307insA	R117C	Q493X	W1089X	
3849+10kbC>T	R1162X	2813AA>G	S1251N**	Y1092X	G178R	1677delTA	D1152H	

Mutation Detection Rate %	Innogenetics	Luminex	Reference (number of alleles tested)
Denmark	93.51	94.10	[3] (n=678)
UK	86.64	87.74	[4] (n=11,402)
Spain	72.71	75.44	[3] (n=1,356)
Argentina	75.92	76.38	[9] (n=440)
Italy	69.27	69.44	[1] (n=3,524)
Belgium	83.59	82.26	[8] (n=2,114)

Immunogenetics mutation panel contains only those mutations in the shaded panel; Luminex mutation panel contains the mutations in both the shaded and unshaded panels, apart from those indicated by a double asterisk (**). Innogenetics mutation panel also includes I148T, recently recognised as a low-penetrance allele and not listed here.
**American College of Medical Genetics (ACMG)-recommended variant.[6]*

Figure 2: Histogram of Wild-type and Mutant Net Signals of a Variant Obtained from an Individual Heterozygous F508del *CFTR* DNA Sample



Expressed as median fluorescence intensity (MFI). The polythymidine tract of intron 8 shows a 5T/9T genotype.

40 are known mutations and three are recommended interference benign variants of F508del (F508C, I507V and I506V).[6] I148T is not included in the Luminex panel as it has been recognised as a low-penetrance allele.[7] In the study, when a new substitution was detected with the Luminex and not with the Innogenetics method, a *CFTR* exonic sequencing, processed by means of BDT3.1/313xl (Applied Biosystems, US) was performed and allowed confirmation of the change. Among discordant samples, three benign variants (F508C, I507V and I506V) identified by the Luminex method in a heterozygous state were confirmed by *CFTR* sequencing.

Post-validation Experience with the Luminex Platform

Following the successful validation procedures described above, about 400 screening tests using the Luminex platform have been performed in routine analyses in our laboratory.

The Luminex method advantageously favours an increased mutation detection rate in different countries across Europe. In Denmark, the European country with the highest relative frequency of F508del mutation,[3] a higher mutation detection rate is obtained. In the UK, a differential mutation detection rate of 1.1% is advantageously recorded (see *Table 1*).[4] A difference of 2.73% between mutation detection rates of the two panels is obtained in Spain.[3] A higher sensitivity is confirmed in a non-European Spanish-speaking population such as Argentina.[9] Among the mutations tested with the Luminex platform, four are common (relative frequency higher than 2.5%) in the following European or Mediterranean countries: R1066C, 3.1% in Portugal; S549R, 2.6% in Algeria; 1677delTA, 3.8% in South of Bulgaria and 2.8% in Turkey; and R347H, 2.8% in Turkey.[3] R352Q is found with a relative frequency of 1% in Lombardy, an Italian region near Milan.[3] A higher sensitivity of the Luminex test is also recorded in the general Italian population.[3] A further extension of the Luminex mutation panel containing 12 additional changes, notably three (S1251N, Q552X, 711+5G>A) of the four missing mutations that are exclusively tested in the Innogenetics panel, is currently being validated in our laboratory. Based on the 2007 annual data report available from May 2009 from the CF Belgian Registry,[8] inclusion of the three mutations will enable increasing the detection rate of 1.27% for the Luminex platform and quite similar sensitivities will then be reached for both methods in Belgium (see *Table 1*).

Finally, due to the large number of mutations found in the *CFTR* gene and their variable frequency among different ethnic and racial groups, it has been difficult to develop a screening assay that covers all mutations and is suitable for all populations. Our experience with the Luminex platform is that this fully automated, semi-quantitative, highly specific and robust platform including ACMG-recommended mutations and variants[6] has been tailored for populations, such as central Europeans, in which migration and miscegenation have increasingly arisen. The platform favours detection of twice as many *CFTR* mutations. Extension of the mutation panel to include Q552X, 711+5G>A, S1251N and nine other mutations, currently under validation in our laboratory, will place Luminex xTAG as the most sensitive platform for routine CFTR molecular diagnosis in European countries. ■

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