# Reverse hybridization enables fast and reliable detection of 25 common CF mutations

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## BACKGROUND AND OBJECTIVES

For cystic fibrosis newborn screening (CFNBS) two reliable but indirect markers, immunoreactive trypsinogen (IRT) and pancreatitis associated protein (PAP), are available for quantitative risk assessment using dried blood spots from newborn screening cards. Sweat testing represents the gold standard for CF diagnosis following a conspicuous newborn screening result, but is of limited reliability in preterm neonates. CFTR mutation analysis using the newborn screening cards allows early diagnosis of CF with high accuracy. Moreover, the well-known clinical and economic benefits of early CF diagnosis as well as the availability of CFTR mutation-specific therapies also highlight the validity of early knowledge of the CFTR genotype. Therefore, a fast and simple assay to screen for common CF mutations using newborn screening cards would be beneficial.

## MATERIALS AND METHODS

We performed analysis of 25 common CFTR mutations using the CFcheck EU-25 kit (Astra Biotech) using 66 samples with known genotype as determined by Sanger sequencing.
DNA extraction was performed within 1 to 14 days after blood collection using either the QIAmp DNA blood mini kit (QIAgen) or the Astra Biotech Multi DNA kit for dried blood spots.
DNA samples were stored at -20 °C. Concentration and quality of extracted DNA was determined spectrophotometrically.

### Principle of reverse hybdrization

Detection of 25 common CFTR mutations using the CFcheck EU-25 kit (Astra Biotech) is based on reverse hybridization.
Fluorescent targets were generated in two consecutive multiplex PCR reactions.
Pooled PCR products were then hybridized to a microarray slide containing probes corresponding to wild type and mutant CFTR alleles. Signal intensities were measured with a laser scanner. CFTR genotypes were assigned based on the position of the detected signals on the microarray slide.

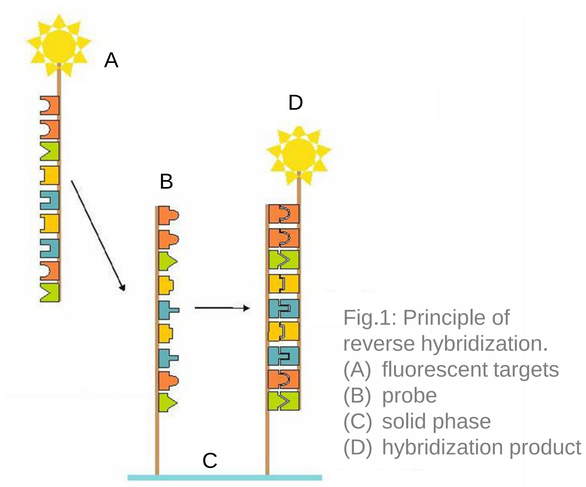


Fig.1: Principle of reverse hybridization.
(A) fluorescent targets
(B) probe
(C) solid phase
(D) hybridization product

### Microarray Analysis

Scanning was performed with the ScanRI microarray scanner (Perkin Elmer). Each slide contains 4 subarrays (Fig. 2) and each subarray contains a wild type, a mutation and a control region. Probes are oligonucleotides representing wild type or mutant CFTR sequences of the certain gene loci. A mutation is assumed to be given if the signal intensities in the mutation fields are at least half as high as the signal intensities in the corresponding wild type fields in at least 2 out of 4 subarrays. Signal intensities were analysed using the CFcheck software.

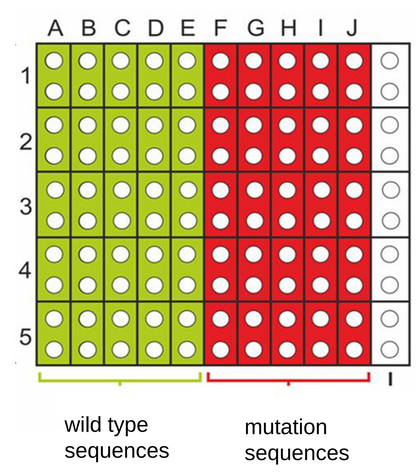


Fig. 2: Subarray from the slide

## RESULTS

### Microarray Scanning

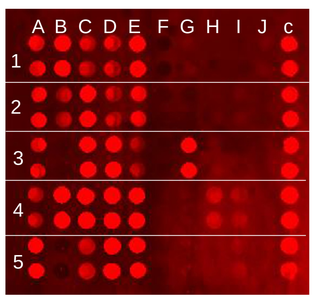


Fig. 3: Microarray of sample with F508del/F508del genotype. The signal on position G3 in the mutation field corresponds to the F508del mutation. The absent signal in the corresponding wild type field B3 and B5 indicate homozygosity. Additional signals in the mutation field were excluded as mutations due to low signal intensity.

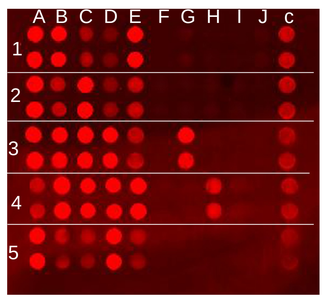


Fig. 4: Microarray of sample with F508del/R553X genotype. The signals on position G3 and H4 in the mutation field besides signals in both corresponding wild type fields (B3 and C4) indicate compound heterozygosity for F508del/R553X.

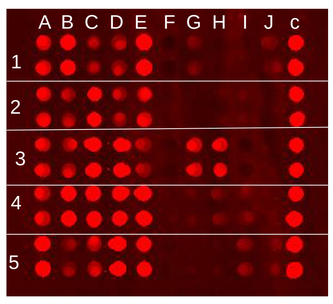


Fig. 5: Microarray of sample with F508del/G551D genotype. The signals on position G3 and H3 in the mutation field besides both corresponding signals in the wild type field B3 and C3 indicate compound heterozygosity for F508del/G551D.

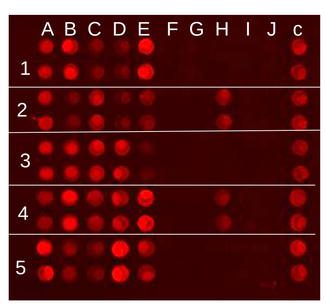


Fig. 6: Microarray of sample with G542X/R553X genotype. The signals on position H2 and H4 in the mutation field besides the corresponding signals C2 and C4 in the wild type field indicate compound heterozygosity for G542X/R553X.

### Quality of mutation detection

| | positive test result T+ | negative test result T- | sum |
|---|---|---|---|
| **mutation present M+** | 113 TP (true positives) | 0 FN (false negatives) | 113 |
| **mutation not present M-** | 7 FP (false positives) | 3180 TN (true negatives) | 3187 |
| **sum** | 120 | 3180 | 3300 |

Table 1: Fourfold table of the mutation detection.

The 66 samples contained:
- 50 samples from patients with two CFTR mutations which were part of the kit‘s panel
- 11 samples from patients with two CFTR mutations of which only one was part of the kit‘s panel
- 3 wild type samples (no CFTR mutation)
- 2 samples from carriers (only one heterozygous CFTR mutation)

Altogether, 113 mutations (M+) had to be detected in the study cohort based on the known genotype (50 samples with 2 detectable mutations + 11 samples with 1 detectable mutation + 2 carriers). In this study, 120 mutations were detected (T+) including 7 falsely detected mutations (T+|M-). These 7 false positive mutation signals were detected in 5 patients in addition to their correctly detected 2 mutations. The additionally detected mutations were I507del in 2 cases (1x heterozygous, 2x homozygous) and R347H (heterozygous) in 2 cases. The sum of 3300 in table 1 arises from 25 detectable mutations on 2 alleles of 66 samples (25*2*66 = 3300). Thus, assuming 113 detectable mutations (M+), 3187 (M-) mutations had to be excluded. Given 120 detected mutations (T+), only 3180 mutations (T-) were excluded in this study.

Therefore, the quality of mutation detection is as follows:
- Sensitivity P(T+|M+) = TP/M+ = 113/113 = 1.0
- Specificity P(T-|M-) = TN/M- = 3180/3187 = 0.998
- Positive predictive value (PPV) P(M+|T+) = TP/T+ = 113/120 = 0.942
- Negative predictive value (NPV) P(M-|T-) = TN/T- = 3180/3180 = 1.0

## SUMMARY AND CONCLUSION

All present CFTR mutations were correctly detected with the kit (sensitivity 1.0). All mutations that could be excluded by the kit, were correctly excluded (NPV 1.0). In a few cases, mutations that were not present according to sanger sequencing, were detected with the kit (specificity 0.998). Therefore, not all detected mutations were actually present (PPV 0.942). All false positive detected mutations occured in cases with mutations on the same exon (e.g. I507del in cases with F508del; R347H in cases with R347P/other). This is most likely due to unspecific hybridization to probes with similar sequences. Unspecific hybridization may be avoided by more stringent washing of slides in order to reduce the number of samples that require repeat analysis. Detection of 25 common CFTR mutations using the CFcheck EU-25 kit represents a sensitive, reliable and fast method for CF diagnosis using newborn screening cards and may complement current CFNBS programs in a multi-tier CFNBS approach.

**Literature**
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