REPORT

Performance of Xpert MTB/RIF Version G4 assay

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Project: 7210

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Technical and Financial Agency:

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Background

As part of the routine product improvement cycle, minor changes were made to the Xpert MTB/RIF assay during the course of the last 12 months to address the following points:

1. During the early phase of rolling out Xpert, a number of sites reported error rates >5%. The assay has therefore been optimized to increase robustness and reduce the occurrence of non-reportable results mainly through the reduction of Signal Loss Detection errors (Error 5011).
2. During the demonstration projects carried out by FIND, a small number of false-resistant results for rifampin susceptibility were encountered (1). In areas where the prevalence of rifampin resistance was low, a significant fraction of all rifampin susceptibility results were false-resistant. A root cause analysis was carried out to determine the cause of this, and software and reagent changes were made to further improve the accuracy of testing.

The new software and cartridge combination, called G4, have been tested analytically at UMDNJ and evaluated clinically in comparison to the existing cartridge at the 5 study sites listed below.

Cepheid will introduce Xpert MTB/RIF kits outside the US with the updated ADF in the December 2011 timeframe.

Partner institutions

- Center for Emerging & Re-Emerging Pathogens
  New Jersey Medical School, Department of Medicine
  University of Medicine and Dentistry New Jersey – New Jersey, US (UMDNJ)
  Principal Investigator: David Alland
- Forschungszentrum Borstel – Borstel, Germany (Borstel)
  Principal Investigator: Elvira Richter
- Institute of Infectious Diseases and Molecular Medicine
  Faculty of Sciences, University of Cape Town
  National Health Laboratory Service – Cape Town, South Africa (UCT)
  Principal Investigator: Mark Nicol
- FIND Uganda – Kampala, Uganda (FIND)
  Principal Investigator: Ajay Thirumala
- Special Treatment Institution of the Main Medical Department
  Ministry of Justice, Republic of Azerbaijan – Baku, Azerbaijan (STI)
  Principal Investigator: Rasim Tahirli
- Instituto de Medicina Tropical "Alexander von Humboldt"
  Universidad Peruana Cayetano Heredia – Lima, Peru (UPCH)
  Principal Investigator: Eduardo Gotuzzo

Summary of findings

- Significant reduction of non-reportable results for Xpert MTB/RIF G4 assay (G4).
- Signal loss error (5011 errors) virtually eliminated.
- Modified Probe B provides improved rifampin detection in the event of annealing temperature fluctuations.
- Improved detection of probe E delay mutants.
- High sensitivity and specificity for TB rifampin resistance detection retained. No significant differences compared to G3.
- Continued post-marketing surveillance important to confirm enhanced performance in larger sample size.

Analytical performance

The Xpert MTB/RIF version G4 assay incorporates modifications summarized in Table 1.

Table 1: **Summary of modifications**	
Fluidics	Modified fluidics to improve robustness and reduce error rate
Assay Settings	Modified background subtraction range Modified valid rpoB Ct range for probes D and E Modified valid SPC Ct range Modified thresholds for probes A, C, D and E
PCR Cycling	Shortened PCR2 anneal time
Beads	Added fluorescent tracer for refined probe check control
Probe B	Modified molecular beacon sequence/quencher

The limit of detection (LOD) for the modified G4 assay was established as described before (2) , and was not found to significantly deviate from the previous assay versions. The LOD point estimate for BCG is 145.4 CFU/ml with a 95% confidence interval ranging from 115.1 CFU/ml to 218.7 CFU/ml.

The probe B sequence was slightly modified so that the probe/wild-type target hybrid was more stable at elevated anneal temperatures. This was to mitigate against false Rif-R results arising from increases in the anneal temperature. The increased robustness for probe B is shown in the graph below. As the anneal temperature increases to 67°C the probe B Ct remains in line with the others, i.e., it does not become the latest probe (highest Ct). Only at 68°C does probe B become later than the others and the resulting Ct difference still does not exceed the cut-off. In summary, the G4 assay is unlikely to generate false-resistant rifampin susceptibility calls in the event of annealing temperature abnormalities. This distinguishes it from the G2 and G3 assays which are at risk for this type of error.

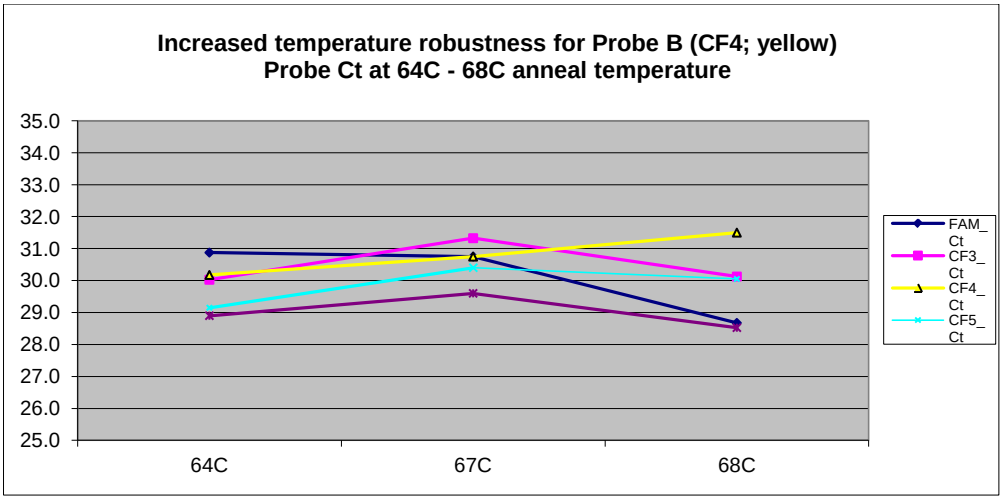

Figure 1. Increased temperature robustness for Probe B

In order to assess the accurate detection of mutations in the rpoB Rifampin Resistance determining region, DNA from 13 clinical isolates representing 7 distinct Rif-Resistant mutations was used to evaluate the Xpert MTB/RIF Assay. DNA samples were run in triplicate at concentrations of 300 copies/ml. Positive (wild type H37Rv) and negative controls were included in this study.

As shown in Table 2, the assay correctly reported Rifampin-Resistance for 38 of 39 replicates. One replicate (*rpoB* 533ccg) was reported Rif-Resistance indeterminate. The wild-type controls H37RV DNA and the negative controls were correctly reported.

Table 2: Detection of *rpoB* mutations

Probe	*rpoB* Mutation	Concentration (copies/rxn)	Replicates Tested	MTB Detected	Rif Resistance Detected	Rif INDETERMINATE Detected	ΔCt Max
A	511 ccg	300	3	3	3	0	29.7
B	516 tac	300	3	3	3	0	5.8
		300	3	3	3	0	5.2
	516 gtc	300	3	3	3	0	12
		300	3	3	3	0	12.9
D	526 cgc	300	3	3	3	0	8.5
		300	3	3	3	0	8.8
	526 gac	300	3	3	3	0	23.9
		300	3	3	3	0	24.2
E	531 ttg	300	3	3	3	0	25.2
		300	3	3	3	0	25.4
	533 ccg	500	3	3	2	1	5.4
		500	3	3	3	0	5.7
H37Rv	WT	300	3	3	0	0	1.5
Neg	N/A	N/A	3	0	0	0	N/A

A separate experiment was performed that compared G2 and G4 versions for detecting particularly challenging probe E mutants. In this study, four mutant strains were grown to an OD of 0.6 and serial ten-fold dilutions made. In the graph below these dilutions are designated -1, -2, -3, -4, and -5. The two assays were run on identical samples for each strain and the delta Ct for probe E compared (only -4, -3, and -2 dilutions were tested with G2). Strains 39 and 166 G4 results had 1 and 2 errors, respectively.

The minimum probe delta Ct for a rifampin resistance call has been increased relative to that used in the G2 version. The graph shows that the reporting of rifampin resistance was significantly improved using the G4 assay compared to the G2 assay.

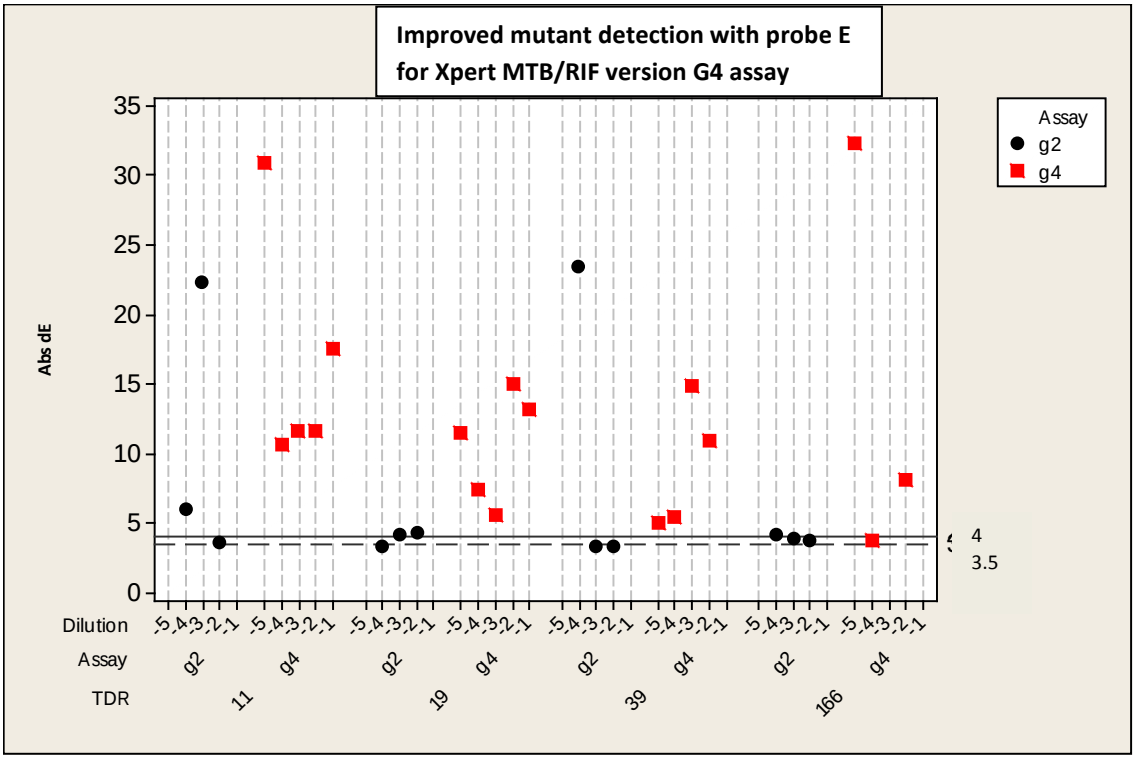

Figure 3. Improved mutant detection with probe E

In summary, the Xpert MTB/RIF G4 assay was developed to increase assay robustness and mitigate against potential false resistant rifampin susceptibility results. The analytical studies described above show that:

- The G4 assay reduces the risk of false rifampin susceptibility results due to anneal temperature abnormalities.
- The limit of detection for G4 does not deviate from previous assay versions.
- Mutation detection with G4 is equivalent to G3 for multiple mutations.
- G4 easily detects Probe E mutants that are difficult to detect with the G3 version.

Clinical performance

This clinical study involved testing specimens of TB suspects from five separate collections of archived specimens from participants from geographically diverse regions in South America, Europe and Africa These specimens had been collected previously as part of other studies conducted by FIND or specifically for this study and were collected in compliance with national and local regulations.

Sputum from 233 primarily TB positive patients stored frozen at less than -70°C were tested in Borstel alongside another 184 frozen, TB positive pellets collected and shipped from the Tropical Medicine Institute Alexander von Humboldt, Universidad Peruana Cayetano Heredia (UPCH) in northern Lima, Peru. All specimens in Borstel were tested on the Xpert MTB/Rif G4 assay.

Two sites in Africa enrolled patients specifically for this study. In Uganda at the FIND-NTRL laboratory in Kampala, during consecutive enrolment of patients from Mulago Hospital, 30 patients' fresh sputum were tested on both the G4 assay and the G3 assay. In South Africa at the University of Cape Town Institute for Infectious Diseases and Molecular Medicine, 100 patients' fresh sputum were tested during consecutive enrolment from May – July 2011. Another 118 patients' enrolled between November 2010 and May 2011 had their unprocessed sputa frozen and stored at less than -70°C and later tested on both the G4 assay and the G3 assay. Recruitment for all patients took place at a clinic at Khayelitsha, an informal settlement in Cape Town.

231 patients were enrolled consecutively in Baku, Azerbaijan for this study. The first spot sputum from each patient was collected and tested fresh on the G3 assay locally. The second and third spot sputa from each patient were frozen and sent to Borstel for testing on G4. For all per patient analysis where more than one test was available for the G4 assay, a single sputum per patient was selected randomly for inclusion before analysis.

The reference standard across all sites included at least one LJ and at least one MGIT culture as well as confirmation of MTB species by Capillia, GenoType MTBDRplus or GenoType Mycobacterium CM/AS. Conventional Rifampicin DST was done from either LJ proportion or MGIT and in a few cases, Hain MTBDR only. Sputa discordant between Xpert MTB/Rif and conventional testing were sequenced for final diagnosis. 6 patients found to be diagnosed as TB and started on treatment despite being smear and culture negative were excluded from the analysis.

The G4 assay performance is summarized in Table 4.

Table 3: Per Patient Performance of Xpert MTB/Rif G4 assay by Country

	Borstel, Germany	Baku, Azerbaijan	Kampala, Uganda	Cape Town, South Africa	All
Sensitivity in culture positive cases (C+)	97.7% (388/397) [95.7% - 98.8%]	81.2% (82/101) [72.5% - 87.6%]	100.0% (6/6) [61.0% - 100.0%]	90.4% (66/73) [81.5% - 95.3%]	93.9% (542/577) [91.7% - 95.6%]
Sensitivity in smear-positive, culture-positive cases (S+C+)	99.4% (321/323) [97.8% - 99.8%]	94.7% (54/57) [85.6% - 98.2%]	100.0% (4/4) [51.0% - 100.0%]	98.0% (50/51) [89.7% - 99.7%]	98.6% (429/435) [97.0% - 99.4%]
Sensitivity in smear-negative, culture-positive cases (S-C+)	87.7% (50/57) [76.8% - 93.9%]	63.6% (28/44) [48.9% - 76.2%]	100.0% (2/2) [34.2% - 100.0%]	72.7% (16/22) [51.8% - 86.8%]	76.8% (96/125) [68.7% - 83.3%]
Specificity in smear-negative, culture-negative cases (S-C-)	100.0% (12/12) [75.7% - 100.0%]	96.4% (106/110) [91.0% - 98.6%]	100.0% (24/24) [86.2% - 100.0%]	98.4% (120/122) [94.2% - 99.5%]	97.8% (262/268) [95.2% - 99.0%]
Sensitivity in Rifampin-resistant cases (Rif-R)	98.6% (72/73) [92.6% - 99.8%]	100.0% (12/12) [75.7% - 100.0%]	0 cases	100.0% (2/2) [34.2% - 100.0%]	98.9%[1] (87/88) [93.8% - 99.8%]
Specificity in Rifampin-sensitive cases (Rif-S)	100.0% (304/304) [98.8% - 100.0%]	98.4% (62/63) [91.5% - 99.7%]	100.0% (6/6) [61.0% - 100.0%]	100.0% (61/61) [94.1% - 100.0%]	99.8%[2] (433/434) [98.7% - 100.0%]

Collections which had tested both the G4 assay version and the G3 assay version were compared directly. This includes patients from Azerbaijan, South Africa, and Uganda. The comparative performance is shown in Table 4. A site-stratified McNamar's test was used to assess whether a significant difference in performance had occurred. None was found. However, the Xpert MTB/Rif G4 version of the assay correctly diagnosed six more TB patients than did the G3 while inaccurately diagnosing two non-TB patients. The only Rif discordant not to-date resolved by sequencing, had mixed G4 results from two sputa samples tested – one Xpert Rif sensitive and the other Xpert Rif resistant – which may indicate a mixed infection or sample mix up.

Table 4: Overall Performance Xpert MTB/Rif of G3 assay versus G4 assay

	Sensitivity in C+	Specificity in S-C-	Rif-R Sensitivity	Rif-S Specificity
G3	82.6% (142/172) [76.2% - 87.5%]	98.8% (242/245) [96.5% - 99.6%]	100.0% (12/12) [75.7% - 100.0%]	100.0% (127/127) [97.1% - 100.0%]
G4	86.0% (148/172) [80.1% - 90.4%]	98.0% (240/245) [95.3% - 99.1%]	100.0% (12/12) [75.7% - 100.0%]	99.2% (126/127) [95.7% - 99.9%]
McNamar's Test p-value	0.51	0.88	0.82	0.95

[1] Xp Rif Sens / DST Rif Resistant Discordants: sequencing of the rpoB region was done for 4 cases and 3/4 were resolved; 1 additional sequencing resulted in mixed infection and this patient was excluded from the analysis

[2] Xp Rif Resistant / DST Rif Sens Discordants : sequencing of the rpoB region was done for 9 cases and 8/9 were resolved

The rate of non-reportable or non-determinate (ND) results was defined as the number of tests with errors, invalid or indeterminate results among all tests performed including repeated tests on the same sputum and within the same patient (Table 5). A Mantel-Haenszel Chi-Square test stratified by site was used to evaluate whether a statistically significant improvement had occurred. The Xpert MTB/RIF version G4 assay showed a significantly lower ND rate for case detection compared to the G3 assay (0.4% non-determinate for G4 and 5.4% for G3, respectively). This drop in ND rate was due to an elimination of Signal Loss Detection errors (error 5011) for the new assay version. 25 of 34 ND were 5011 errors for G3 compared to 0 of 5 ND for G4. Additionally, the RIF specific ND rate was not highly affected by the assay changes going from 0.5% in the G3 assay to 0.9% in G4.

Table 5: Xpert non-determinant (ND) rate

	Xpert ND Rate for Case detection	Xpert ND Rate for Rifampicin susceptibility
G3	5.4% (34[i]/627)	0.5% (1/210)
G4	0.4% (5[ii]/1252)	0.9% (6/692)
Chi-Square Test p-value	<0.001	0.69

In summary, the changes made to the assay have not negatively impacted its performance. The number of non-reportable results has been significantly reduced.

1. Boehme CC, Nicol MP, Nabeta P, Michael JS, Gotuzzo E, Tahirli R, et al. Feasibility, diagnostic accuracy, and effectiveness of decentralised use of the Xpert MTB/RIF test for diagnosis of tuberculosis and multidrug resistance: a multicentre implementation study. Lancet. Apr 30;377(9776):1495-505.
2. Helb D, Jones M, Story E, Boehme C, Wallace E, Ho K, et al. Rapid detection of Mycobacterium tuberculosis and rifampin resistance by use of on-demand, near-patient technology. Journal of clinical microbiology. Jan;48(1):229-37.

[i] 73.5% (25/34) 5011 Error ; 17.6% (6/34) invalid ; 2.9% (1/34) 5006 Error ; 2.9% (1/34) 5007 Error ; 2.9% (1/34) 2014 Error.
[ii] 60% (3/5) 5007 Error; 20% (1/5) 2014 Error; 20% (1/5) invalid.