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Early View

Original article

Compatibility of a novel filter paper-based bio-safe sputum transport kit with Line Probe Assay for diagnosing drug-resistant tuberculosis: a single-site evaluation study

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Take home message. The adoption of bio-safe 'TB Concentration & Transport' kit by Microscopy Centres can potentially overcome the challenge of transporting infectious sputum to Central laboratories and provide Universal-DST services to TB subjects residing in remote areas.

Abstract.

Background. Near-patient access to appropriate tests is a major obstacle for the efficient diagnosis of Tuberculosis (TB) and associated drug resistance.

Methods. We recently developed the 'TB Concentration & Transport' kit for bio-safe, ambient-temperature transportation of dried sputum on *Trans*-Filter, and the 'TB DNA Extraction' kit for DNA extraction from *Trans*-Filter for determining drug resistance by DNA sequencing. In the present study, we evaluated the compatibility of Kit-extracted DNA with Hain's Line Probe Assays (LPAs), which are endorsed by National TB programmes for the detection of drug resistance in sputum collected from presumptive Multi-drug resistant TB patients (n=207).

Results. *Trans*-Filter-extracted DNA was seamlessly integrated with the LPA protocol (Kit-LPA). The sensitivity of Kit-LPA for determining drug resistance was 83.3% for rifampicin (95% Confidence Interval [CI]: 52, 98%), 77.7% for isoniazid (95% CI: 52, 94%), 85.7% for fluoroquinolones (95% CI: 42, 100%) and 66.6% for aminoglycosides (95% CI: 9, 99%), with a specificity range of 93.7% (95% CI: 87, 97) to 99.1% (95% CI: 95, 100) using phenotypic drug susceptibility testing (DST) as a reference standard. A high degree of concordance was noted between results obtained from Kit-LPA and LPA [99% to 100% (κ value: 0.83-1.0)].

Conclusions. This study demonstrates successful integration of our developed kits with LPA. The adoption of these kits across Designated Microscopy Centres in India can potentially overcome the existing challenge of transporting infectious sputum at controlled temperature to centralized testing laboratories and can provide rapid near-patient cost-effective 'Universal DST' services to TB subjects residing in remote areas.

Introduction.

Tuberculosis (TB) is one of the leading causes of death worldwide and India accounts for 26% of the world's total TB burden [1]. Around 500,000 new cases of rifampicin (RIF) resistant TB were noted in 2019, of which 78% were multidrug resistant TB (MDR-TB) [1]. The rapid increase in the number of drug resistant TB cases has been further exacerbated by the ongoing COVID-19 pandemic which has created critical gaps in diagnosing and providing care to TB patients [2]. This highlights the necessity of widespread drug susceptibility testing (DST) for implementing patient-centric anti-TB regimens [1]. However, DST implementation is a major challenge in primary healthcare centres (PHCs), particularly in remote geographical areas of India and other high burden countries, where DST facilities are restricted to centralized laboratories such as National/Intermediate reference laboratories (NRLs/IRLs). At present, DST services are extended to patients residing in remote areas by sample transport under temperature-controlled and bio-safe containment conditions [3]. In view of these impediments, a safe and robust modality for sputum transportation from Designated Microscopy Centres (DMCs) or District Tuberculosis Centres to centralized laboratories is a priority requirement of the National TB Elimination Programme (NTEP) [3].

The technology for the detection of drug resistant TB is moving towards rapid molecular-DST (Mol-DST) from conventional culture-based DST approaches. The World Health Organization (WHO) endorsed tests for drug resistance testing include the Xpert MTB/RIF assay, Xpert Ultra, Truenat test and Line Probe Assays (LPA) [4]. At present, Xpert and Truenat tests provide information only on rifampicin resistance while LPA, namely GenoType® MTBDR*plus* VER 2.0 (first line LPA) and GenoType® MTBDR*sl* tests VER 2.0 (second line LPA) both from Hain LifeSciences, Nehren, Germany, are comprehensive

molecular tests for MDR-TB and XDR-TB, respectively. In India, Hain's LPAs (henceforth referred as LPA) are mainly used in the NTEP program and are recommended for use in only direct smear-positive sputum specimens and culture isolates of smear-negative sputum samples [5]. In India in 2019, 3,46,282 first line LPA and 72,748 second line LPA tests were performed, compared to only 16,399 culture-DST tests [3]. This indicates the scalability of LPA testing. However, the use of LPA is restricted to NRLs/IRLs and certified laboratories (n=64) with sophisticated facilities and trained manpower that are not available at the DMC level [3], which raises the logistic challenge of transporting infectious sputum from remote are as to the testing laboratory and also poses biosafety issues.

To address this unmet need, we have recently developed the 'TB Concentration & Transport' kit for collecting bacteria present in sputum on a *Trans*-Filter device [6]. This bio-safe *Trans*-Filter can be shipped at ambient temperature and DNA can be extracted at the DST laboratory [6] using the 'TB DNA Extraction' kit (Supplementary material: Figure S1). The primary objectives of this study were to evaluate the compatibility of Kit-extracted DNA with LPA and to compare the performance (diagnostic accuracy) of Kit-LPA with that of the WHO-endorsed LPA test. The secondary objectives of the study were to evaluate the bio-safety of the *Trans*-Filter device and to obtain performance feedback from the scientists and technicians who have used the kits.

Materials and Methods.

Study subjects and design. This study was designed and supervised by Translational Health Science and Technology Institute (THSTI), Faridabad, and the All India Institute of Medical Sciences (AIIMS), New Delhi. Presumptive MDR-TB/XDR-TB patients were included in the study according to the Programmatic Management of Drug Resistant TB Guidelines, namely

belonging to one or more of the following categories: TB patients found positive on any follow-up sputum smear examination during treatment with first-line drugs, including treatment failures, drug resistant-TB patients contacts, previously treated TB patients, recurrent TB patients (TB diagnosed after completing a course of TB treatment), and patients retrieved after loss to follow-up [5]. All patients were enrolled after Institutional Ethical Clearance at the National Institute of Tuberculosis and Respiratory Diseases (NITRD, NITRD/EC/2017/0228) and Translational Health Science and Technology Institute [THSTI, THS 1.8.1/(70)]. Sample size (n=234) was estimated based on 85% power, alpha of 5% and positivity of 44% vs. 32% of Kit-extracted DNA-based sequencing vs. MGIT-DST for determination of MDR-TB (unpublished data). The study was performed in a double–blind manner from June 2018 through February 2019 on prospectively collected fresh sputum samples in Outpatient Department (OPD) at NITRD (Appendix S1). This study adhered to the Standards for Reporting of Diagnostic Accuracy (STARD) guidelines and a completed checklist is included (Appendix S2).

Sample collection and processing. One sputum sample was collected from each patient. Firstly, a loopful of sputum from this sample was used to perform Ziehl-Neelsen (ZN) staining at OPD, smears were observed and graded as recommended by NTEP guidelines [7]. Only smear-positive patients were enrolled in the study, as NTEP recommends direct LPA testing only on smear-positive sputum (Figure 1). All smear-positive samples were transported to Microbiology Department of NITRD where an aliquot of sputum was processed using 'TB Concentration & Transport' kit followed by LPA using DNA extracted from *Trans*-Filter (henceforth cited as Kit-LPA) using 'TB DNA Extraction' kit. Another aliquot was processed for bio-safety assessment as described below. The leftover sputum sample was processed by N-acetyl L-cysteine (NALC)-sodium hydroxide (NaOH) method

for LPA, Xpert MTB/RIF, MGIT culture in a double-blind manner. A unique 4-digit code was assigned to each sample for different tests (4 codes per sputum sample) and each test was performed by separate laboratory personnel. The results were decoded and analysed at the completion of the study.

Line Probe Assay. All sputum samples were decontaminated by NALC-NaOH method [8]. DNA was extracted from deposits obtained after decontamination using GenoLyse® DNA Extraction Kit VER 1.0 (Hain Lifesciences, Nehren, Germany) followed by PCR amplification, and reverse hybridization (using GT-Blot 48 system, Hain Lifesciences) using 1st line LPA and 2nd Line LPA as per the manufacturer's instructions [9, 10].

Kit-LPA. All sputum samples were processed using 'TB Concentration & Transport' kit [6]. Briefly, 400 µl of 'Dissolving solution' was added to 100 µl of sputum and incubated for 30 minutes at room temperature. Thereafter, 300 µl of liquefied sputum (equivalent to ~60 µl of neat sputum) was filtered through the *Trans*-Filter followed by the addition of 'Sterilizing solution' and 'Stabilizing solution' [6]. Then DNA was extracted from the *Trans*-Filter (that were stored at room temperature for 2-5 days; Figure 1, filter F1) using 'TB DNA Extraction' kit [6]. Kit-extracted DNA was directly used in PCR amplification followed by reverse hybridization steps of LPA as per the manufacturer's instructions [9, 10].

Xpert MTB/RIF assay. The NALC-NaOH processed sputum samples were also subjected to Xpert assay. Briefly, 0.5 ml of processed sputum sample was taken and 1.5 ml volume of

sample reagent was added, vortexed and then incubated for 15 min at room temperature. Then 1 ml of this suspension was used for Xpert [11].

Mycobacteria Growth Indicator Tube (MGIT) culture. All decontaminated sputum samples were subjected to MGIT culture (Figure 1). MGIT tubes (Becton, Dickinson, USA) showing positive signal were subjected to ZN staining (for the presence of cords) for presumptive detection of *M. tuberculosis* complex. The presence of *M. tuberculosis* was confirmed by SD BIOLINE TB Ag MPT64 kit Rapid test (Standard Diagnostics, South Korea).

MGIT-DST. DST was performed for all *M. tuberculosis* culture-positive samples. Briefly, 0.5 ml MGIT positive *M. tuberculosis* culture was inoculated into MGIT containing different drugs i.e. RIF (1 µg/ml), isoniazid (INH, 0.1µg/ml), levofloxacin (FLQ, 1 µg/ml) or kanamycin (AMN, 2.5 µg/ml) as described [12, 13].

Bio-safety evaluation. Bio-safety assessment culture was performed on n=207 smear-positive sputum samples. All sputum samples were processed using the 'Transport kit' as described previously [6]. Briefly, 400 µl of 'Dissolving solution' was added to 100 µl of sputum and incubated for 30 minutes at room temperature. Thereafter, 300 µl of liquefied sputum (equivalent to ~60 µl of neat sputum) was filtered through the *Trans*-Filter followed by the addition of 'Sterilizing solution' and 'Stabilizing solution'. The *Trans*-Filter was taken out from the device using forceps and the membrane was placed into a MGIT culture tube. The MGIT culture tubes were incubated at 37 ºC for up to 42 days (Figure 1, filter F2).

Feedback questionnaire. Briefly, based on feedback obtained from the users during development and pilot evaluation of these kits [6], we prepared two structured questionnaires to collect feedback from the evaluating site (NITRD, New Delhi) in the present study.

The questionnaire for the scientist aimed at (i) collecting information about the kits packaging, kits components (whether the kits have all the components in proper condition without any leakage and with proper labelling at the time of receiving); (ii) to collect viewpoint on the user manual (whether it is simple and easy to understand and descriptive enough to follow or any improvement is required); and (iii) to obtain the feedback of the scientist on ease of the use of the kits, its user friendliness, benefits and disadvantages, and suggestions for further improvement of the kits. The questionnaire for the technician included items to obtain the viewpoint of technician on training (for the use of kits), user manual, ease of use of kits and feedback on improvement in the kits, if required (Annexure 3).

Kit performance and statistical analysis. Data from all presumptive MDR-TB subjects was collected using the proforma prepared for the study (Appendix S4). Samples with incomplete data, invalid and indeterminate results were excluded from the study (invalid and indeterminate results were defined as per manufacturer's instructions [9, 11]). The analysis was done at 3 levels for drug resistance detection (Figure 2); (i) Comparison of Kit-LPA/LPA against MGIT-DST. In this analysis, we excluded results that were TB negative by Kit-LPA/LPA and/or culture negative by MGIT and "Indeterminate" results for the respective drug targets in Kit-LPA/LPA and/or samples with missing MGIT-DST results due to culture contamination (Figure S2 and S3), (ii) Comparison of Kit-LPA against LPA. In this analysis, we excluded results that were TB negative in either or both LPA and Kit-LPA and "Indeterminate" results for the respective drug targets by LPA and/or Kit-LPA (Figure S4), (iii) Comparison of Kit-LPA/LPA against Xpert. In this analysis, we excluded results that

were negative by Xpert and/or TB negative by Kit-LPA/LPA, and "Indeterminate" results for RIF resistance in Xpert and/or LPA/Kit-LPA (Figure S5).

The sensitivity of Kit-LPA was calculated as [True positives] / [True positives + False negatives]; wherein true positives were defined as samples identified as drug resistant by both Kit-LPA and phenotypic DST, and false negatives are samples which were missed by Kit-LPA but scored as resistant by phenotypic DST. Specificity was defined as [True negatives] / [True negatives + false positives]; where true negatives are samples that were sensitive by both Kit-LPA and phenotypic DST, and false positives were samples showing mutations by Kit-LPA but called as sensitive by phenotypic DST. Sensitivity and specificity estimates of LPA and Xpert were also calculated similarly. Concordance between Kit-LPA and LPA results was calculated as [True positives + true negatives] / [total number of samples]and the degree of concordance/agreement was measured by Cohen's kappa (κ) (https://www.graphpad.com/quickcalcs/kappa1/). McNemar'schi-square test was used to compare the performance of Kit-LPA vs. LPA (https://epitools.ausvet.com.au/mcnemar). Sample size was estimated using G*Power 3 software [14].

Results.

Study participants. Three hundred and twenty-nine participants were screened in the present study, of which 207 subjects who were smear-positive were enrolled in the study (Figure 2). They were in the age range of 4–97 years (including 16 children, age between 3-17 years) and around 69% (144/207) patients were males. The most common clinical symptoms were cough (~95%, 197/207), weakness (~90%, 187/207), loss of appetite (83%, 172/207), weight loss (~80%, 165/207) and fever (~61%, 127/207, Table S1). The HIV status of 8/207 patients were available and all of them were HIV-negative.

Performance of LPA and Kit-LPA *vs.* MGIT-DST. The detection of wild type/mutant alleles of drug resistance genes by Kit-LPA and LPA was assessed using MGIT-DST as a gold standard (Figure 3). The sensitivity of Kit-LPA was 83.3% (95% CI: 52, 98%) and 77.7% (95% CI: 52, 94%) for detecting RIF and INH resistance, respectively; and was quite comparable to that of LPA which was 83.3% for both RIF (95% CI: 52, 98%) and INH (95% CI: 59, 96%) resistance (Table 1). For detecting resistance to fluoroquinolones (FLQ) and aminoglycosides (AMN), the sensitivity of both Kit-LPA and LPA was 85.7% (95% CI: 42, 100%) and 66.6% (95% CI: 9, 99%), respectively (Table 1). The specificity range was quite similar for all 4 drugs; the specificity of Kit-LPA ranged from 93.7% (95% CI: 87, 97%) to 99.1% (95% CI: 95, 100%) and for LPA from 94.5% (95% CI: 88, 99%) to 100% (95% CI: 97, 100%) (Table 1). These results demonstrate that the use of *Trans*-Filter-extracted DNA enables similar test outcomes as the LPA for the rapid determination of drug resistance profiles (Figure 3).

Performance of Kit-LPA. Using LPA as a gold standard, the Kit-LPA showed a sensitivity and specificity in the range of 96.5-100% and 98.7-100%, respectively, for all 4 drugs (Table 2, 95% CI values are included). There was no significant difference in the performance of Kit-LPA vs. LPA ($p=0.48$ to 1.0 for all 4 drugs) and a concordance of 98.8-100% (κ value 0.83-1.0) was noted (Table 2). In case of discrepancy, both Kit-LPA and LPA were repeated.

In a stratified analysis of LPA results from 11/16 children for whom these results were available, a concordance of 100% (κ value 1.0) was noted between Kit-LPA and LPA.

Comparative performance of Kit-LPA, LPA and Xpert MTB/RIF. Kit-LPA and LPA had a concordance of 97.1% (κ value 0.85) and 98.2% (κ value 0.90), respectively, with Xpert for RIF resistance determination.

Bio-safety assessment. None of the sputum samples processed by the 'Transport kit' were *M. tuberculosis* positive after 6 weeks of incubation of *Trans*-Filter inoculated in MGIT culture (Figure 4). Efficient disinfection of sputum samples was achieved irrespective of the smear grade status of the sample (Figure 4). These results indicated 100% success in disinfection of sputum samples by the 'Transport kit'.

Discussion.

In resource-limited high TB burden countries, a simple, rapid and cost-effective method for sputum transport from DMCs and PHCs to NRLs/IRLs remains an immediate and unmet need to this day [3]. In India under NTEP, efforts are being made to link postal and courier services for sputum transport from peripheral centres to laboratories having culture and molecular diagnostics facilities to provide 'Universal DST' services [3]. However, a requirement for triple layer packaging, maintaining cold chain during transport and speedy delivery are daunting challenges in the field [15].

In view of these challenges, the WHO's target product profile (TPP) has highlighted several desirable parameters for 'sputum transport methods' that include compatibility with *M. tuberculosis* detection assays, simple equipment-free procedure, bio-safety and stability during transportation [16]. To overcome the aforementioned challenges, we recently developed the 'TB Concentration and Transport' kit for use at DMCs/PHCs to enable safe

and ambient temperature transport of sputum on *Trans*-Filter to a higher-level laboratory for further investigation. The Transport kit fulfils all the requirements of the TPP except for 'compatibility with culture methods' which requires viable bacteria. In contrast, this kit fulfils the bio-safety criterion, it achieved a disinfection of a minimum 8-log of *M. tuberculosis* [6] thereby minimizing bio-hazard exposure to workers during processing and transportation of samples from DMCs/PHCs. The level of disinfection provided was comparable to that provided by the 'Sample Reagent' in the Xpert MTB/RIF assay [6]. The kit is compatible with Mol-DST approaches, such as DNA sequencing [6] and LPA (present study). In this study, the compatibility of the kit was assessed with the WHO-recommended LPA detection of RIF and INH resistance in place of phenotypic DST [4] and this points to the increasing scope of molecular DST tests to replace culture-based approaches in the near future.

A comparison of the features of our kit with those of other sputum transport kits currently available in the market [16] indicates our Transport kit to be more cost-effective (INR 100 or USD 1.40 per sample) as compared to others, in addition to being bio-safe and compatible with dry sputum transport (Table 3). The 'TB DNA Extraction' kit is also attractively priced at INR 100 (USD 1.36) per sample as compared to the currently used kit in LPA i.e. GenoLyse® DNA Extraction kit (INR 138 or USD 1.88/ sample) and other commercially available kits for DNA isolation such as QIAamp DNA Mini Kit (Qiagen, USA, INR 191 or USD 2.60/sample) and PrimeXtract extraction kit (Long Horn Vaccines and Diagnostics, USA, INR 262 or USD 3.57/sample).

The sensitivity and specificity of Kit-LPA for RIF, INH, FLQ and AMN were in the range of 66.6%-85.7% and 93.7%-99.1%, respectively, which was quite similar to that of LPA using MGIT-DST as a reference standard (Table 1), with a concordance value of 91.5%-98.3% (κ value 0.64-0.74) for MDR-TB and XDR-TB (Table 1). The sensitivity of LPA (both Kit-LPA and LPA) for detecting drug resistance in our study (~67% to ~86%) was

somewhat lower to the pooled sensitivity reported previously in a meta-analysis (86% to 96%) [4]. A possible reason for this lower sensitivity may be a comparatively smaller sample size, where a small discrepancy in classifying a sample as sensitive or resistant, results in a greater difference in sensitivity. The discrepancies noted between MGIT-DST and LPA/Kit-LPA results are summarized in Table S2 and might be attributed either to the presence of mutations outside the resistance determining region probed in LPA [17-19], or disputed or inferred mutations (which show low level resistance in MGIT-DST) [20, 21], or heteroresistance as reported earlier [6, 19, 22].

The most important finding of this study was that the overall performance of Kit-LPA was quite similar to that of LPA (Figure 3) and it is noteworthy that a minimal level of discordance was observed between these two tests (n=3, Table S2). The discrepancies were rechecked by repeating Kit-LPA and LPA tests. The interpretation of LPA as per manufacturer's instructions is based on the detection of band intensity (wild type or mutant probe) being greater than or equal to that of the 'Amplification Control' band [9, 10]. A slight difference in band intensity between Kit-LPA and LPA could have caused a difference in interpretation and thereby leading to discordance.

The feedback from the scientists and laboratory technicians revealed the biggest benefits of kits to be their ease-of-use, bio-safe sputum transport on filter paper at ambient temperature and easy integration of Kit-extracted DNA with LPA protocol. The feedback also highlighted the minimal training requirement for Transport kit procedure to laboratory technicians. This feedback will be helpful for assessing these kits in a feasibility study under field settings in the future.

Strengths and limitations of the study. We have demonstrated in the present study that DNA extracted from *Trans*-Filter is compatible with 1st line and 2nd line LPA for the detection of MDR-TB and XDR-TB. These findings are especially noteworthy when seen in the context of the current scenario of NTEP's DST programme where in Hain's LPA is used for detection of MDR-TB and XDR-TB (depending on RIF susceptibility result) for all TB cases diagnosed by Xpert. Mol-DST tests require sophisticated laboratory infrastructure with bio-safety compliance, and they are not easy to implement at the peripheral level. The bio-safe sputum transport kit reported here is highly suitable for use in lower level set ups such as DMCs and PHCs, as it eliminates the use of equipment for sputum concentration and minimizes the risk of aerosol generation. The transport filter combines smoothly with the 'TB DNA Extraction' kit to provide pure *M. tuberculosis* DNA from sputum for integration with various molecular DST tests. The second noteworthy finding was that the performance of Kit-LPA was highly concordant with LPA for all 4 drugs and with Xpert for determining RIF resistance. Thirdly, the feedback from laboratory technicians and scientists highlighted the benefit of *Trans*-Filter for bio-safe dried sputum transport and integration of extracted DNA with LPA. A limitation of the study was that sputum *Trans*-Filters were not transported from remote areas.

Conclusions. Our findings have laid the foundation for the deployment of these kits towards achieving the goal of 'Universal DST'. Due to its ease of use, cost-effectiveness and patient accessibility, the sputum processing and transport technology described here has the potential to transform the diagnostic supply chain by providing near-patient cost-effective 'Universal DST' services to TB subjects residing in remote geographical areas of India. This technology has the potential to positively impact DST not only in India, but also in other high burden countries where sample transportation is a formidable barrier to the widespread

implementation of DST. Kit-LPA is poised for evaluation infield settings under NTEP for the detection of MDR-TB and XDR-TB.

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Conflict of interest. AG and NKG manufactured and provided the TB Concentration & Transport' and 'TB DNA Extraction' kits and were not involved in any aspect of conducting the study and analysing the results. DA, RKG, AV, VPM, RS, AG, NKG, SH and JST are joint inventors in an Indian Provisional Patent application named 'Apparatus and method for processing a sample for rapid diagnosis of tuberculosis and safe transport of bacteria' (Patent application number- 201811042155).

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Table 1. Performance of Kit-LPA and LPA vs. MGIT-DST

for the detection of MDR-TB and XDR-TB.

Kit-LPA vs. MGIT-DST				LPA vs. MGIT-DST			
Drug[a] (No. of Samples)[*]	**Sensitivity % (95% CI)[b]**	**Specificity % (95% CI)**	**Con %[c] (κ)**	**Drug[a]**	**Sensitivity % (95% CI)**	**Specificity % (95% CI)**	**Con % (κ)**
RIF (n=130)	83.3 (52, 98)	96.6 (91, 99)	95.3 (0.74)	**RIF (n=128)**	83.3 (52, 98)	97.4 (93, 99)	96.1 (0.77)
INH (n=130)	77.7 (52, 94)	93.7 (87, 97)	91.5 (0.67)	**INH (n=128)**	83.3 (59, 96)	94.5 (88, 99)	91.5 (0.73)
FLQ (n=122)	85.7 (42, 100)	95.7 (90, 99)	95.1 (0.64)	**FLQ (n=124)**	85.7 (42, 100)	95.7 (90, 99)	95.1 (0.64)
AMN (n=122)	66.6 (9, 99)	99.1 (95, 100)	98.3 (0.66)	**AMN (n=124)**	66.6 (9, 99)	100 (97, 100)	99.2 (0.79)

[a]RIF, Rifampicin; INH, Isoniazid; FLQ, Fluoroquinolones; AMN, Aminoglycosides
[b]CI, Confidence interval
[c]Con, Concordance with phenotypic DST result; κ, Cohen's kappa coefficient
*The details of samples are provided in Figures S2 and S3.

Table 2. Performance of Kit-LPA for the detection of MDR-TB and XDR-TB*.

		Kit-LPA vs. LPA		
Drug[a] (No. of samples)[@]	**Gene target**	**Sensitivity % (95% CI)[b]**	**Specificity % (95% CI)**	**Concordance % (κ)[c]**
RIF	*rpoB* (n=197)	100 (85, 100)	98.8 (96, 100)	98.8 (0.95)
INH	*katG* (n=196)	96.5 (82, 100)	100 (98, 100)	99.4 (0.98)
	inhA (n=197)	100 (40, 100)	100 (98, 100)	100 (1.0)
FLQ	*gyrA* (n =179)	100 (80, 100)	98.7 (96, 100)	98.8 (0.94)
	gyrB[#] (n=179)	Not estimable	100 (98, 100)	100 (Not estimable)
AMN	*rrs* (n=179)	100 (48, 100)	98.8 (96, 100)	98.8 (0.83)
	eis (n=179)	100 (2, 100)	100 (98, 100)	100 (1.0)

*Using LPA as a gold standard.

[a]RIF, Rifampicin; INH, Isoniazid; FLQ, Fluoroquinolones; AMN, Aminoglycosides

[b]CI, Confidence interval

[c]κ, kappa value

[#]There was no mutant sample in *gyrB* drug target, therefore sensitivity and kappa value of concordance could not be estimated.

[@]The details of samples are provided in Figure S4.

Table 3. Comparison of 'TB Concentration & Transport' kit *vs.* other commercially available transport kits.

Product	**Manufacturer**	**Material transported**	**Assessed downstream applications***	**Advantages**	**Limitations**	**Cost per sample**
TB Concentration & Transport kit (used in present study)	Advanced Microdevices (mdi), Ambala, India	Dried sputum on filter	NAATs [LPA (present study), PCR, Sanger sequencing [6]]	Spill proof, ambient temperature transport, bio-safe	Field testing is pending	INR 100/ USD 1.36
PrimeStore Molecular Transport medium	Longhorn Vaccines and Diagnostics, San Antonia, TX, USA	Liquid sputum in tube	NAATs [Xpert [23], LPA[24], RT-PCR[25], NGS [26-29]], transcriptome analysis [30]	Bio-safe	Risk of sample spillage	INR 300/ USD 4.10
FTA card (recently discontinued)	Whatman, GE Healthcare Life Sciences, Pittsburgh, PA, USA	Spotted sputum on filter	NAATs [LPA, PCR [31, 32]]	Spill proof, ambient temperature transport	Not bio-safe	INR 165/ USD 2.25
GenoCard	Hain Lifescience, GmbH, Nehran, Germany	Spotted sputum on filter	NAATs [LPA, PCR [31, 32]]	Spill proof, ambient temperature transport	Not bio-safe	Not available for sale in India

Figure legends.

Figure 1. Workflow of the study.

Figure 2. Workflow of sample analysis in the study.

Figure 3. Comparison of Kit-LPA and LPA vs. MGIT-DST for detection of MDR-TB and XDR-TB.

Figure 4. Bio-safety culture results of samples processed by the 'TB Concentration & Transport' kit. *Negative for *M. tuberculosis* by SD Bioline MPT64 Ag Rapid test.

S**upplemental material.**

Figure S1. Evaluated kits: 'TB Concentration & Transport' and 'TB DNA Extraction' Kits.

Figure S2. Workflow of sample analysis (Kit-LPA vs. MGIT) for drug resistance detection. **M. tb*: *Mycobacterium tuberculosis*, confirmed by AFB-positive smear and SD BIOLINE TB Ag MPT64 kit Rapid test (in MGIT-culture).

Figure S3. Workflow of sample analysis (LPA vs. MGIT) for drug resistance detection. **M. tb*: *Mycobacterium tuberculosis*, confirmed by AFB-positive smear and SD BIOLINE TB Ag MPT64 kit Rapid test (in MGIT-culture).

Figure S4. Workflow of sample analysis (Kit-LPA vs. LPA) for drug resistance detection. **M. tb*: *Mycobacterium tuberculosis*, confirmed by AFB-positive smear and SD BIOLINE TB Ag MPT64 kit Rapid test (in MGIT-culture).

Figure S5. Workflow of sample analysis (Kit-LPA/LPA vs. Xpert MTB/RIF) for drug resistance detection. **M. tb*: *Mycobacterium tuberculosis*, confirmed by AFB-positive smear and SD BIOLINE TB Ag MPT64 kit Rapid test (in MGIT culture).

Table S1. Clinical characteristics of enrolled participants in the present study.

Table S2. Discordance between results of MGIT-DST, LPA and Kit-LPA.

Appendix S1. Patient Informed Consent Form.

Appendix S2. STARD checklist.

Appendix S3. Questionnaire for evaluating the 'TB Concentration and Transport' kit performance.

Appendix S4. Data collection sheet.

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Figure 1

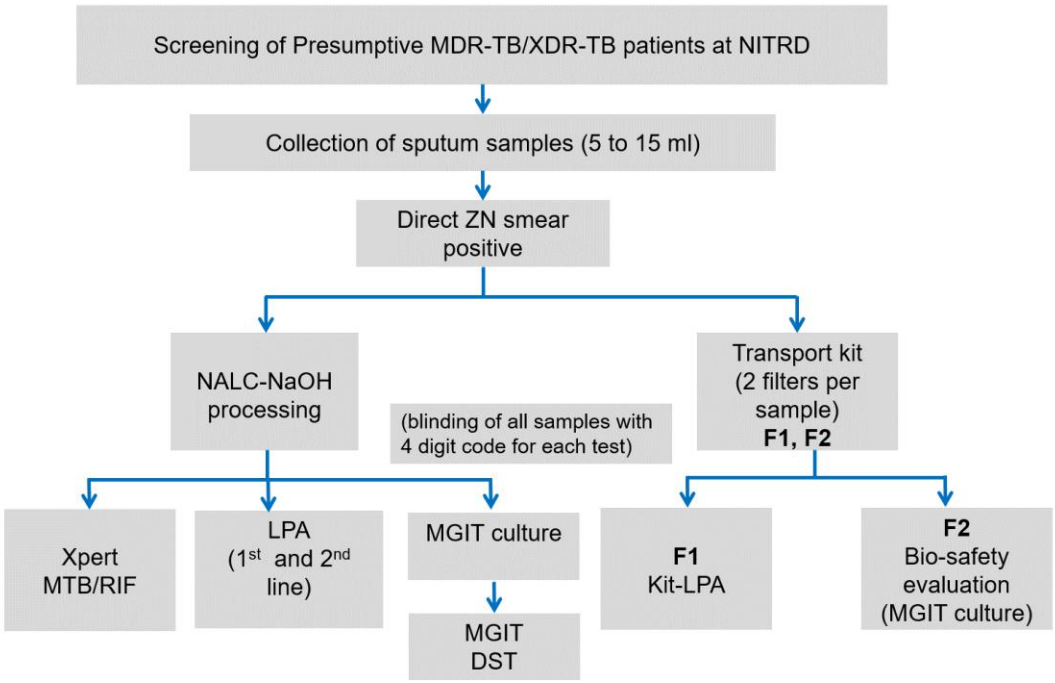
Screening of Presumptive MDR-TB/XDR-TB patients at NITRD
Collection of sputum samples (5 to 15 ml)
Direct ZN smear positive
NALC-NaOH processing
(blinding of all samples with 4 digit code for each test)
Transport kit (2 filters per sample) F1, F2
Xpert MTB/RIF
LPA (1st and 2nd line)
MGIT culture
MGIT DST
F1 Kit-LPA
F2 Bio-safety evaluation (MGIT culture)

Figure 2

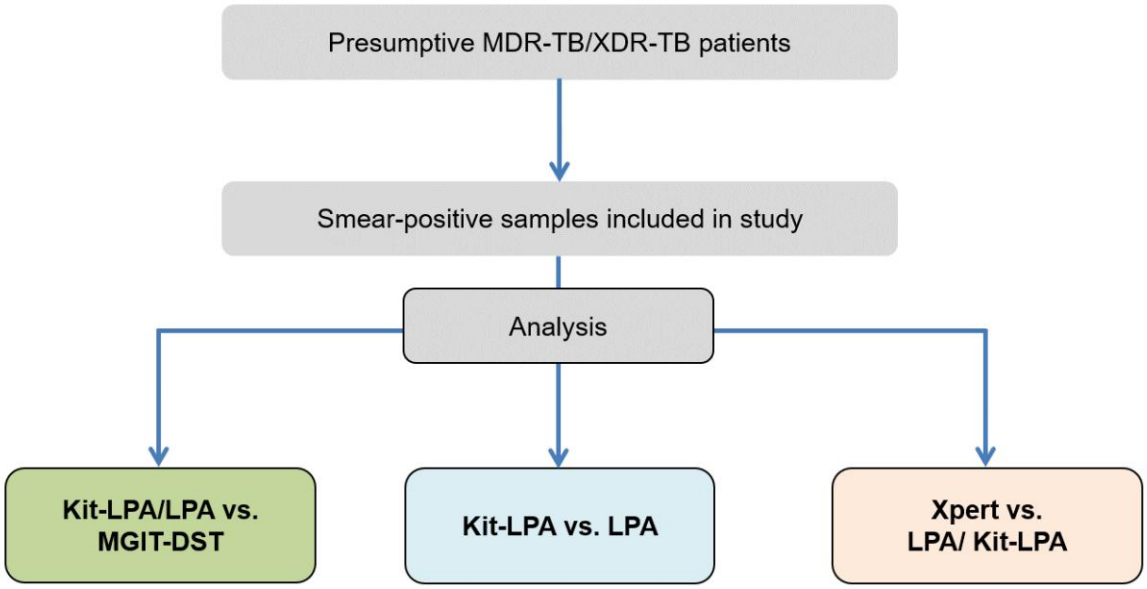
Presumptive MDR-TB/XDR-TB patients
Smear-positive samples included in study
Analysis
Kit-LPA/LPA vs. MGIT-DST
Kit-LPA vs. LPA
Xpert vs. LPA/ Kit-LPA

Figure 3

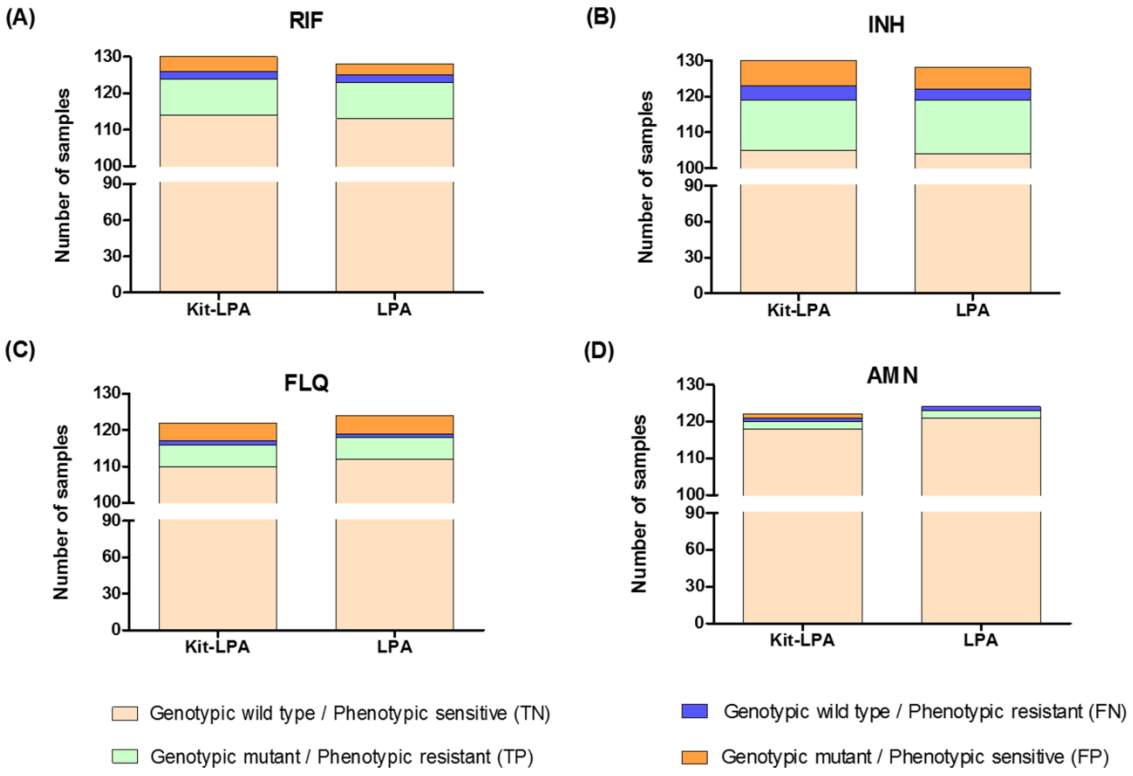
(A)
RIF
(B)
INH
(C)
FLQ
(D)
AMN
Number of samples
130
120
110
100
90
60
30
0
Kit-LPA
LPA
Genotypic wild type / Phenotypic sensitive (TN)
Genotypic wild type / Phenotypic resistant (FN)
Genotypic mutant / Phenotypic resistant (TP)
Genotypic mutant / Phenotypic sensitive (FP)

Figure 4

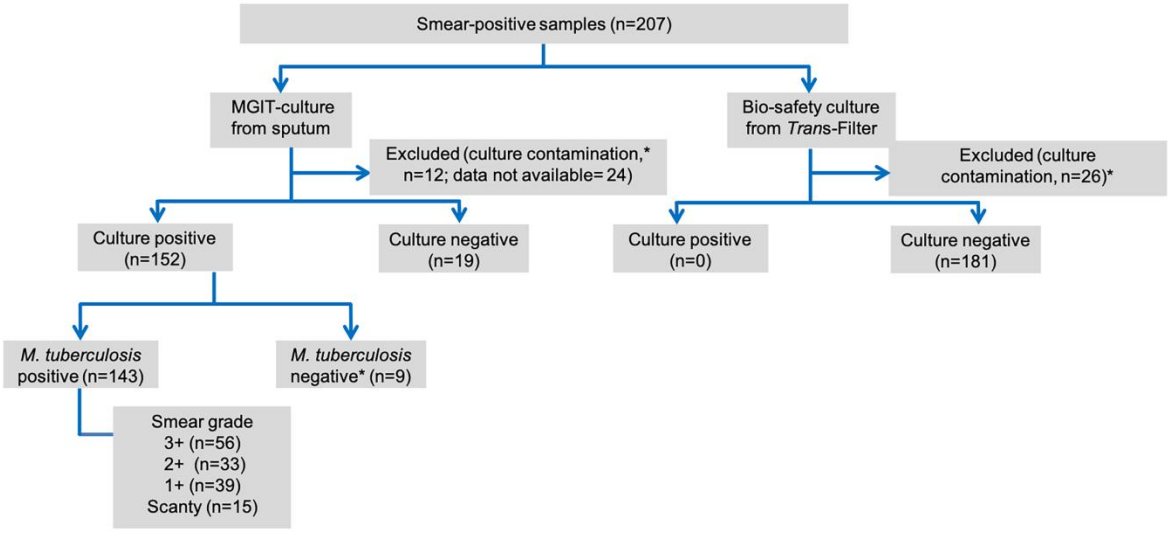
Smear-positive samples (n=207)
MGIT-culture from sputum
Bio-safety culture from *Trans*-Filter
Excluded (culture contamination,* n=12; data not available= 24)
Excluded (culture contamination, n=26)*
Culture positive (n=152)
Culture negative (n=19)
Culture positive (n=0)
Culture negative (n=181)
M. tuberculosis positive (n=143)
M. tuberculosis negative* (n=9)
Smear grade
3+ (n=56)
2+ (n=33)
1+ (n=39)
Scanty (n=15)

Figure S1

Figure S1. Evaluated kits: 'TB Concentration & Transport' and 'TB DNA Extraction' Kits.

Figure S2

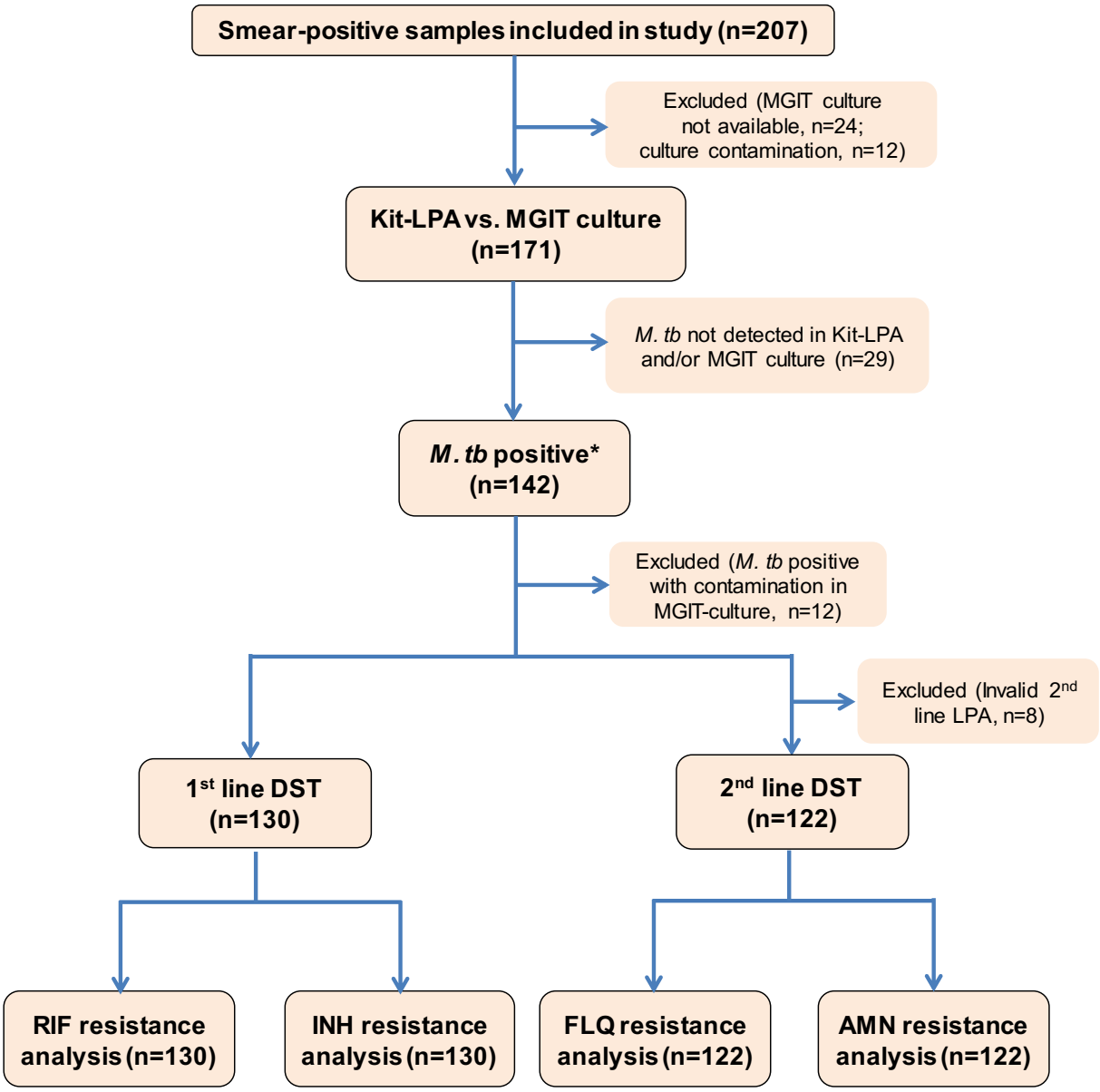

Figure S2. Workflow of sample analysis (Kit-LPA vs. MGIT) for drug resistance detection. **M. tb*: *Mycobacterium tuberculosis*, confirmed by AFB-positive smear and SD BIOLINE TB Ag MPT64 kit Rapid test (in MGIT-culture).

Figure S3

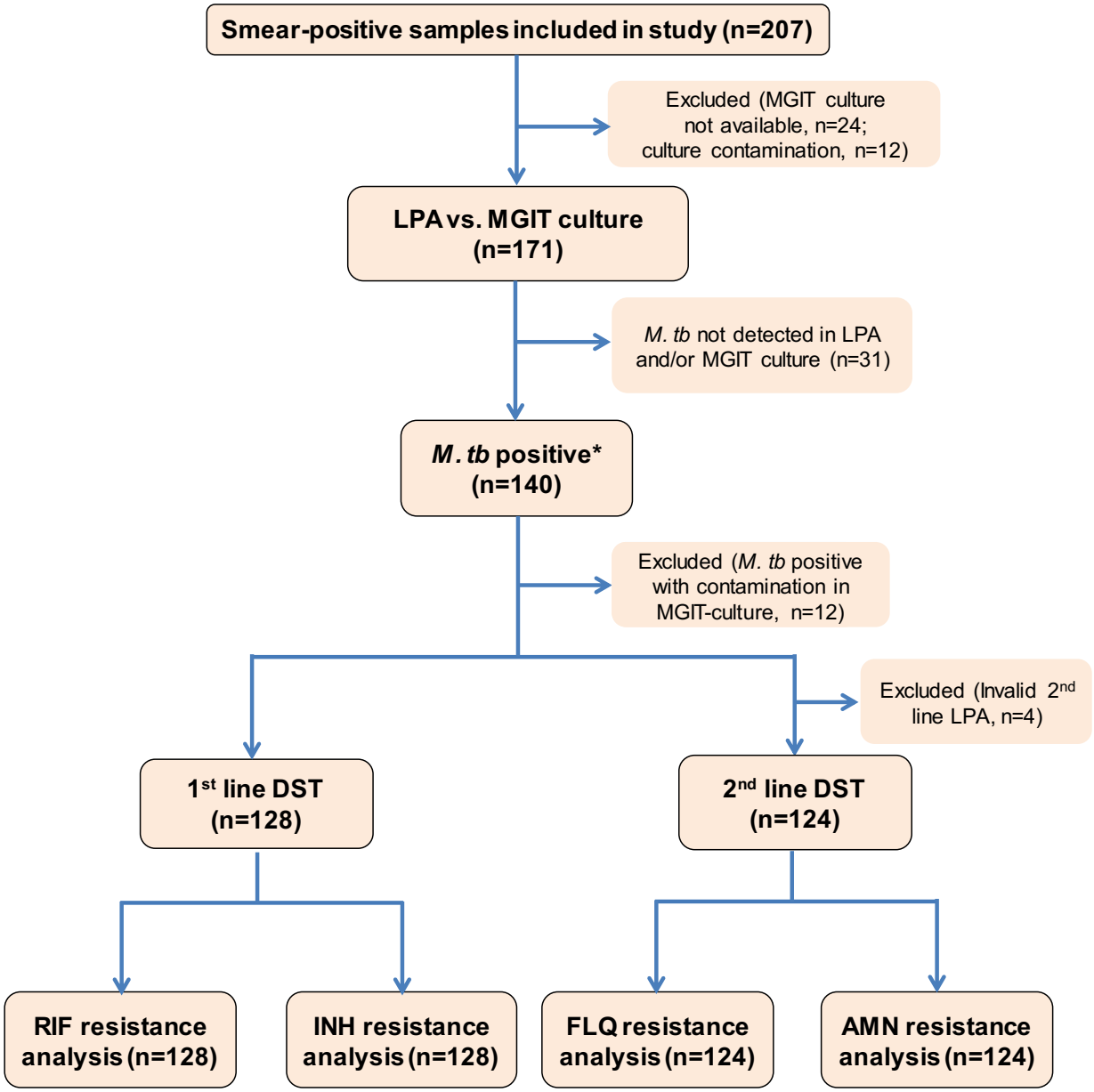

Figure S3. Workflow of sample analysis (LPA vs. MGIT) for drug resistance detection. **M. tb*: *Mycobacterium tuberculosis*, confirmed by AFB-positive smear and SD BIOLINE TB Ag MPT64 kit Rapid test (in MGIT-culture).

Figure S4

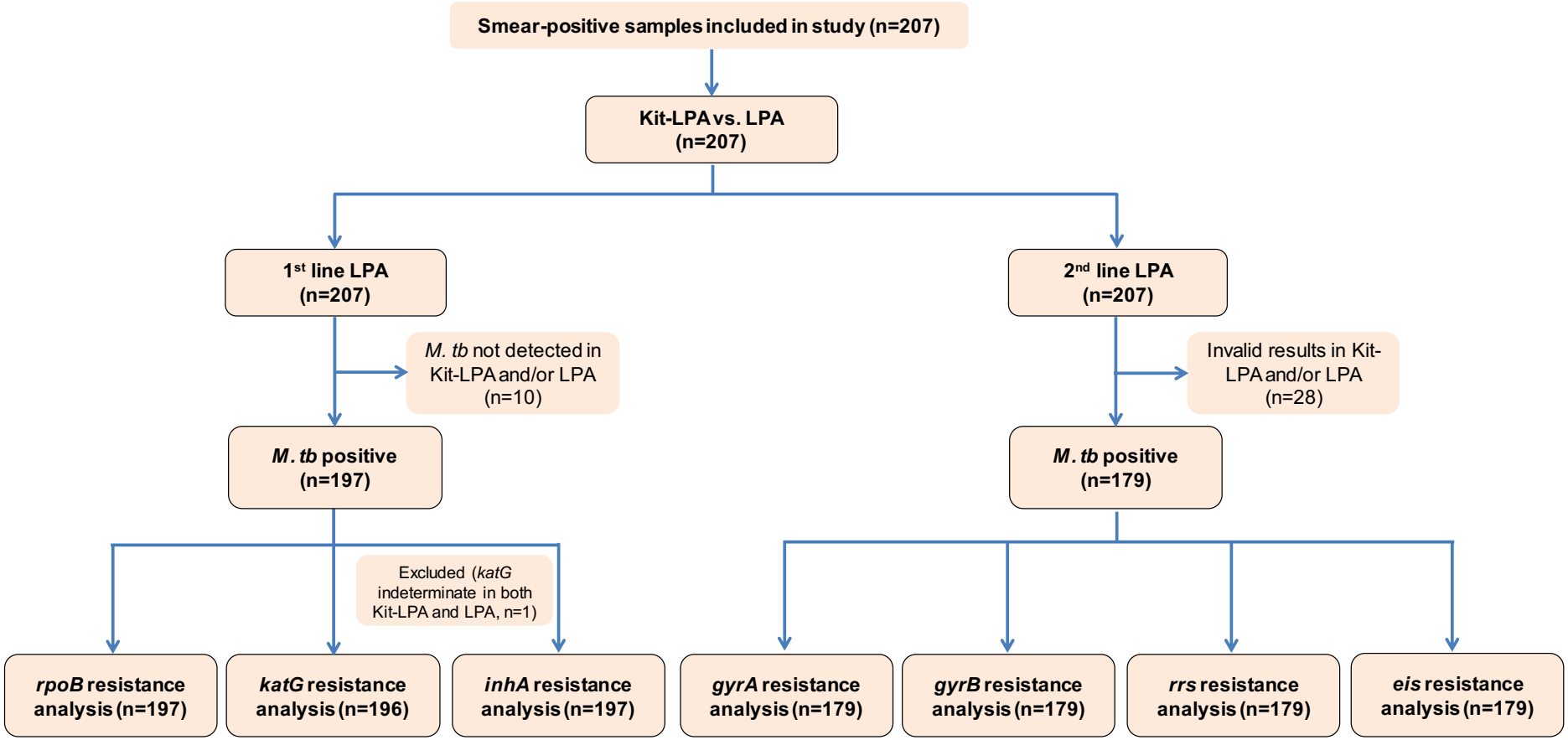

Figure S4. Workflow of sample analysis (Kit-LPA vs. LPA) for drug resistance detection. **M. tb*: *Mycobacterium tuberculosis*, confirmed by AFB-positive smear and SD BIOLINE TB Ag MPT64 kit Rapid test (in MGIT-culture).

Figure S5

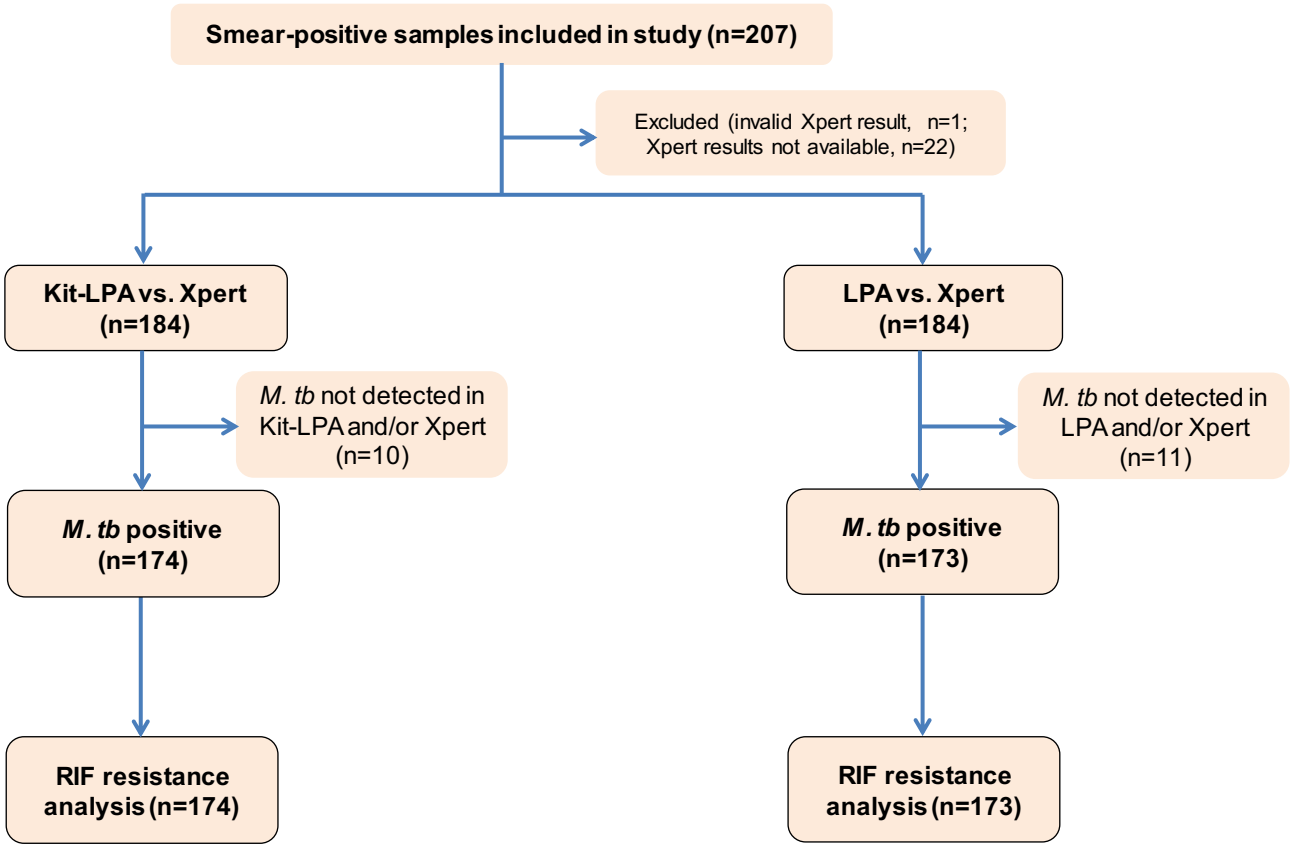

Figure S5. Workflow of sample analysis (Kit-LPA/LPA vs. Xpert MTB/RIF) for drug resistance detection. **M. tb*: *Mycobacterium tuberculosis*, confirmed by AFB-positive smear and SD BIOLINE TB Ag MPT64 kit Rapid test (in MGIT culture).

Table S1. Clinical characteristics of enrolled participants in the present study.

Symptom/ Criterion	**Presumptive MDR-TB/XDR-TB Patients n=207 (%)**
Fever	127 (61.3)
Cough	197 (95.1)
Haemoptysis	31 (4.5)
Night sweats	72 (34.7)
Weakness	187 (90.3)
Loss of appetite	172 (83.1)
Weight loss	165 (79.7)
Abdominal distension	51 (24.6)
Vomiting	69 (33.3)
Lymph node enlargement	9 (4.3)

Table S2. Discordance between results of MGIT-DST, LPA and Kit-LPA.

Drug(Number of samples analysed)*	Sample Id	Results			Discordance		
		MGIT-DST@	Kit-LPA#	LPA#	MGIT-DST vs. Kit-LPA	MGIT-DST vs. LPA	LPA vs. Kit-LPA$
RIF (n=128)	C8743, C2444	R	WT	WT	2	2	0
	C8549, C8495, C7384	S	MUT	MUT	3	3	0
INH (n=128)	C4236, C7384, C6648	R	WT	WT	3	3	0
	C9353	**R**	**WT**	**MUT**	**1**	**0**	**1**
	C3264, C8495, C8743, C4754, C3489, C5579	S	MUT	MUT	6	6	0
FLQ (n=118)	C9652	R	WT	WT	1	1	0
	C3728	**S**	**MUT**	**WT**	**1**	**0**	**1**
	C3742, C8549, C2692, C3489	S	MUT	MUT	4	4	0
AMN (n=118)	C6382	R	WT	WT	1	1	0
	C3728	**S**	**MUT**	**WT**	**1**	**0**	**1**

*This table summarized data of only those samples that had complete results for these 3 tests.

@R, Resistant in MGIT-DST; S, Sensitive in MGIT-DST.

#WT, Wild-type in LPA/Kit-LPA; MUT, Mutant in LPA/Kit-LPA.

$Discordant results between Kit-LPA vs. LPA are highlighted in bold font.

Appendix S1: Patient Informed Consent Form

Sr. No._____ **Date_____**

Patient's Name _____ **Age _____ Sex ___**

I have been explained the details of the study entitled "**Multi-centric validation of 'TB-Detect' and "TB Concentration and Transport' kit and 'TB DNA extraction' kit for the diagnosis of TB and drug resistant TB"** and my questions regarding the study have been answered to my satisfaction in a language understood by me

1. The nature and purpose of the study and its potential risks / benefits and expected duration of the study, and other relevant details of the study have been explained to me in detail.
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal right being affected.
3. I understand that my participation in the study is confidential and that the information collected about me from my participation in this research and sections of any of my medical notes may be looked at by responsible individuals or from regulatory authorities where it is relevant to my taking part in research.
4. I consent to give my sample for the purpose of this study. I understand that on completion of the study or if I withdraw from this study my sample will be destroyed and I understand that if there is any problem with any of the tests of measurement then I will be informed and the report will be kept confidential.

I hereby provide the consent to take part in the study entitled "**Multi-centric validation of** '**TB Detect', 'TB Concentration and Transport' kit and 'TB DNA extraction' kit for the diagnosis of TB and drug resistant TB"**

Signature/Thumb impression of the Patient Signature of the Investigator

Name & Address

In case of any emergency, please contact:

1. Dr. Rohit Sarin and Dr V P Myneedu, NITRD, New Delhi 011-26963335
2. Dr. Jaya Sivaswami Tyagi, AIIMS, New Delhi 011-26594609
3. Dr. Sagarika Haldar, THSTI, Faridabad 0129-2876352

Appendix S2: STARD checklist

Section & Topic	No	Item	Reported on page #
TITLE OR ABSTRACT	**1**	Identification as a study of diagnostic accuracy using at least one measure of accuracy (such as sensitivity, specificity, predictive values, or AUC)	Page # 1- 3 The title and abstract identify the manuscript as a study of diagnostic accuracy.
ABSTRACT	**2**	Structured summary of study design, methods, results, and conclusions (for specific guidance, see STARD for Abstracts)	Page # 3 A structured abstract including objective, methods, results and conclusions are included.
INTRODUCTION	**3**	Scientific and clinical background, including the intended use and clinical role of the index test	Page # 4 and 5 The Introduction focuses on the manuscript in a wider context. It includes a brief review of the key references and the need for the development of the 'TB Concentration and Transport' and 'TB DNA Extraction' kits and evaluation for its compatibility with Who endorsed GenoType MTBDR*plus* and GenoType MTBDR*sl* tests.
	4	Study objectives and hypotheses	Page # 5 The 'TB Concentration and Transport' kit consisting of *Trans*-Filter device was developed to fulfil the need of sputum transport from lower-level laboratories to central laboratories in a bio-safe and a cost-effective manner. The 'TB DNA Extraction' kit was developed to extract DNA from *Trans*-Filter for rapid detection of TB and its associated drug resistance. In the present study, we evaluated the compatibility of above mentioned kits extracted DNA with WHO endorsed GenoType MTBDR*plus* and GenoType MTBDR*sl* tests.
METHODS			

Study design	**5**	Whether data collection was planned before the index test and reference standard were performed (prospective study) or after (retrospective study)	Page # 5 and 6 Data collection was planned before the index test and reference standard were performed. The study was a prospective study performed in a double-blind manner.
Participants	**6**	Eligibility criteria	Page # 5 and 6 Patients belonging to the Presumptive MDR-TB/XDR-TB patient group were included in the study.
	7	On what basis potentially eligible participants were identified (such as symptoms, results from previous tests, inclusion in registry)	Page # 5 and 6 Patients belonging to the Presumptive MDR-TB/XDR-TB patient were included in the study.
	8	Where and when potentially eligible participants were identified (setting, location and dates)	Page # 5 and 6 All patients were enrolled after Institutional Ethical Clearance at the National Institute of Tuberculosis and Respiratory Diseases (NITRD, NITRD/EC/2017/0228) and Translational Health Science and Technology Institute [THSTI, THS 1.8.1/ (70)]. We obtained written informed consent from participants prior to sample collection.
	9	Whether participants formed a consecutive, random or convenience series	Page # 5 and 6 Patients belonging to the Presumptive MDR-TB/XDR-TB group were included consecutively in the study.
Test methods	**10a**	Index test, in sufficient detail to allow replication	Since our study involves validation of the kits, we have given details of the kit(s) protocol in page number 7 which can be sufficiently replicated while using the kit manual/protocol.
	10b	Reference standard, in sufficient detail to allow replication	Page #7 and 8 MGIT Culture and Culture DST were used as a reference standard wherever applicable, which is the conventional gold standard for TB diagnosis. Details are explained in referenced page numbers.
	11	Rationale for choosing the reference standard (if alternatives exist)	NA
	12a	Definition of and rationale for test positivity cut-offs or result	Page # 8 and 9

		categories of the index test, distinguishing pre-specified from exploratory	The TB Concentration and Transport' and 'TB DNA Extraction' kits was assessed for its compatibility with WHO endorsed GenoType MTBDR*plus* and GenoType MTBDR*sl* tests.
	12b	Definition of and rationale for test positivity cut-offs or result categories of the reference standard, distinguishing pre-specified from exploratory	Page # 9 and 10 MGIT Culture was performed by standard NALC-NaOH method. The results were confirmed by using ZN smear and SD BIOLINE TB Ag MPT64 Rapid test (Standard Diagnostics). Then, MGIT-DST was performed from primary culture with standard drug MICs.
	13a	Whether clinical information and reference standard results were available to the performers/readers of the index test	Page # 6 and 7 The clinical information and reference standard results were not available to the performers/readers of the index test as the study was carried out in a double-blind manner.
	13b	Whether clinical information and index test results were available to the assessors of the reference standard	Page # 6 and 7 The clinical information and index test results were not available to the assessors of the reference standard as the study was carried out in a double-blind manner.
Analysis	**14**	Methods for estimating or comparing measures of diagnostic accuracy	Page # 9 and 10 Included in statistical analysis.
	15	How indeterminate index test or reference standard results were handled	Page # 9 and 10, Supplementary Fig S2-S5. Samples with indeterminate results were excluded from the study.
	16	How missing data on the index test and reference standard were handled	Page # 9 and 10, Supplementary Fig S2-S5. Samples with indeterminate results were excluded from the study.
	17	Any analyses of variability in diagnostic accuracy, distinguishing pre-specified from exploratory	Page # 9 and 10 Included in statistical analysis.
	18	Intended sample size and how it was determined	Page # 6. Sample size was estimated based on 85% power, alpha of 5% and positivity of 44% vs. 32% of kit extracted DNA based sequencing vs. MGIT-DST for determination of MDR-TB (unpublished data) using G*Power 3 software.

RESULTS			
Participants	**19**	Flow of participants, using a diagram	Page # 10 and Supplementary Fig S2-S5.
	20	Baseline demographic and clinical characteristics of participants	Page # 10
	21a	Distribution of severity of disease in those with the target condition	NA
	21b	Distribution of alternative diagnoses in those without the target condition	NA
	22	Time interval and any clinical interventions between index test and reference standard	NA
Test results	**23**	Cross tabulation of the index test results (or their distribution) by the results of the reference standard	Page # 10 and 11 (Table 1 and Table 2).
	24	Estimates of diagnostic accuracy and their precision (such as 95% confidence intervals)	Page # 10 and 11 (Table 1 and Table 2).
	25	Any adverse events from performing the index test or the reference standard	NA
DISCUSSION			
	26	Study limitations, including sources of potential bias, statistical uncertainty, and generalisability	Page # 14 and 15 Included under Discussion section.
	27	Implications for practice, including the intended use and clinical role of the index test	Page # 15 The 'TB Concentration & Transport' kit is suitable for use in lower-level laboratories such as DMCs and PHCs as it eliminates the requirement of centrifugation for sputum concentration which requires electricity and carries a potential risk of aerosol generation. It combines smoothly with the 'TB DNA Extraction' kit which provides highly

			pure DNA directly from sputum samples that integrates with molecular diagnostic approaches methods. In the future, the kit-integrated LPA will be evaluated for operational feasibility and performance in field settings under 'NTEP' for the detection of MDR-TB and XDR-TB. Details are included in Discussion section.
OTHER INFORMATION			
	28	Registration number and name of registry	NA
	29	Where the full study protocol can be accessed	NA
	30	Sources of funding and other support; role of funders	Details are given in 'Funding information' section.

Appendix S3

QUESTIONNAIRE FOR SCIENTIST

Scope: This questionnaire is aimed at inviting the Scientist's viewpoint about the packaging, kit components, and user manual related to 'TB Concentration & Transport' Kit in field settings.

Date:

Name of Scientist: Name of DMC:

Education: District:

Experience: State:

(To be filled every time the kit package is received)

Product Lot No.:

		Packaging			Remarks
	A.	Was the package intact when received?	Yes	No	
	B.	Is the package easy to open and use?	Yes	No	
	C.	Is the overall packaging robust to protect kit contents?	Yes	No	
	D.	If package is damaged, please describe.			

		Kit Components			Remarks
	A.	Were all the following components present in the kit?			
		● User Manual	Yes	No	
		● Droppers	Yes	No	
		● TB Transport Devices	Yes	No	
		● SD Tube with powder	Yes	No	
		● Zip Lock Bags	Yes	No	
		● Polyethylene Sheets	Yes	No	
		● 7 solution bottles	Yes	No	
	B.	Was any solution bottle leaking?	Yes	No	
	C.	Were all the components labeled and identifiable?	Yes	No	
	D.	Were the components neatly packed and properly placed in the box?	Yes	No	

(To be filled once at the start of study)

		User Manual			Remarks
	A.	Is the user manual easy to read and understand?	Yes	No	
	B.	Does it have complete information for user?	Yes	No	
	C.	Are all the steps well described?	Yes	No	
	D.	Are the steps mentioned in correct sequence?	Yes	No	
	E.	Are the images shown helpful and informative?	Yes	No	
	F.	Is troubleshooting guide easy to follow?	Yes	No	
	G.	Is troubleshooting guide effective/enough?	Yes	No	
	H.	Are improvements in the manual required? If yes, please specify.			

(To be filled only once at the end of study)

		Overall Feedback			Remarks
	A.	Is the kit user friendly?	Yes	No	
	B.	How many samples did your operator run before he/she felt comfortable processing samples on their own?			
	C.	What do you feel are the biggest benefits of the kit? Please specify.			
	D.	Do you feel there are any disadvantages/negative points of the kit? Please specify.			
	E.	Assuming the cost is affordable, which other aspect(s) of the 'TB Concentration & Transport' kit do you think would be the main barrier(s) for adoption by health workers?			
	F.	How do you think the 'TB Concentration & Transport' kit could be further improved?			

QUESTIONNAIRE FOR TECHNICIAN

Scope: This questionnaire is aimed at inviting the technician's viewpoint about the user manual and daily use activities related to 'TB Concentration & Transport' Kit in field settings.

Date:

Name of Technician: Name of DMC:

Education: District:

Experience: State:

(To be filled once at the start of study)

		Understanding			Remarks
	A.	Was the training given for use sufficient?	Yes	No	
	B.	Is the user manual easy to read and understand?	Yes	No	
	C.	Please specify the improvements you would like to see in the user manual.			
	D.	Were you able to adapt to the use of transport kit?	Yes	No	
	E.	How many samples did you run before you felt comfortable processing samples on your own?			
	F.	How do you think the 'TB Concentration & Transport' kit could be further improved?			

(To be filled once at the end of study)

	How would you rate TB Detect compare to conventional slide method on scale of 0-5 in terms of:	**Points**
A.	Ease of Use	
B.	Operator Fatigue	
C.	Time Saving	

(To be filled every day during the study period)

Date:

	Working	**Remarks**
A.	Total no. of samples performed	
B.	Name of solution bottle that did not deliver drops as required?	
C.	Number of cases where powder in SD tube does not come down after tapping. Please specify.	
D.	Number of cases where dissolving solution was added less or more than black mark. Please specify.	
E.	Number of cases where dissolved sputum could not be filtered completely. Did you proceed with the completion of protocol anyway?	
F.	Number of cases where pre-filter funnel was not attached to device. Please specify.	
G.	Number of cases where filter membrane was not attached to pre-filter funnel. Please specify.	
H.	Number of cases where filter membrane got detached from the holder. Did you pick up the membrane with forceps and transferred it to the bag? Please specify.	

Appendix S4- DATA COLLECTION SHEET

PATIENT DETAILS								
S.NO.	DATE	NAME	PATIENT REGISTRATION NO.	AGE	SEX	ADDRESS	HIV STATUS (If available)	FEVER

CLINICAL SYMPTOMS			CLINICAL SYMPTOMS					
COUGH	BLOOD IN SPUTUM	NIGHT SWEATS	WEAKNESS	LOSS OF APETITE	WEIGHT LOSS	ABDOMINAL DISTENSION	VOMITING	LYMPH NODE ENLARGEMENT

SPUTUM		PATIENT CATEGORY		PATIENT CATEGORY				
QUALITY	QUANTITY	PRESUMPTIVE MDR/XDR	TREATMENT FAILURE	CONTACT TO MDR PATIENT	RELAPSE CASE	DIRECT LED-FM CODE/LAB NO.	DIRECT LED-FM RESULT	MGIT CODE

	MGIT-DST RESULT						DIRECT LPA RESULT					
MGIT RESULT	H	R	K	L	XPERT CODE	XPERT RESULT	DIRECT LPA CODE	*rpoB*	*katG*	*inhA* promoter	*gyrA*	*gyrB*

		KIT LPA RESULT				KIT LPA RESULT				
rrs	*eis* promoter	KIT CODE	*rpoB*	*katG*	*inhA* promoter	*gyrA*	*gyrB*	*rrs*	*eis* promoter	KIT BIOSAFETY RESULT