



## DEMONSTRATION STUDY PROTOCOL

Effectiveness of  
LED-based fluorescence  
microscopy for the  
detection of TB in ZN  
microscopy centers

### STUDY OUTLINE

*Technical and Financial Agency:*

**Foundation for  
Innovative New Diagnostics**

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**Demonstration Study Protocol iLED**

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Confidentiality statement

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## **Background**

Replacing light microscopy with fluorescence microscopy would be one of the immediate options for improving TB case finding in high burden countries. A systematic review by WHO/TDR and FIND has shown that: a) Fluorescence microscopy is, on average, 10% more sensitive than conventional light microscopy. The increased sensitivity is greatest in low grade positives. b) The specificity is comparable. c) Reading a fluorochrome stained smear takes only 25% of the time taken to read a ZN stained smear [1].

The recent application of ultra-bright LED (light emitting diodes) technology to enable inexpensive fluorescence microscopy is a potentially significant advance in TB diagnostics. Zeiss, in a joint development agreement with FIND, has developed a fluorescence microscope promising excellent performance at a most competitive price. Other LED-based approaches, such as the FRAEN After device, designed to attach to a bright field microscope, and the screw-in objective from LWS are or will become available shortly. Recently completed and ongoing studies from a number of groups [2-4] appear to confirm the equivalence of LED-powered microscopes to their much more expensive, conventional fluorescence counterparts. In collaboration with national TB programs and international partner organizations, FIND, will explore the feasibility and impact of scaling up the use of LED fluorescence microscopes to improve TB control.

## **Project Purpose**

The project aims at a) confirming the performance and b) demonstrating the effectiveness of LED-based fluorescence microscopy for detection of tuberculosis using the Primo Star iLED developed by Zeiss in partnership with FIND. The data will be reported to national TB and public health authorities such as the Strategic Technical Advisory Board of the WHO to inform policy decision.

## **Partner Selection**

### Criteria for country selection

- Agreement at National Level (MOU with NTP and/or MOH)
- High-burden of TB
- Low or middle income
- At least 2 countries with high HIV prevalence
- Local presence of FIND or implementing partner
- Representative of global TB situation

### Criteria for selection of microscopy centers

National TB Programs will select the microscopy centers that shall participate in the project as demonstration sites and will base their selection on the following criteria:

- Selected microscopy centers must be accessible by monitors from the respective supervisory site on a daily basis during validation phase and on a weekly or twice per month basis during the implementation phase
- At least 10-15% smear positivity

- At least 1 microscopist present who has undergone formal microscopy training
- For settings with 3 demonstration sites, it is desirable to select 1 high volume (>40 smears per day), 1 medium volume (20-40 smears per day) and 1 low volume microscopy center (approx. 10 smears per day)
- For settings with 2 demonstration sites, at least one of the sites should be a medium volume center
- At least 2 sites with only intermittent power supply will be included (1 located in India, and 1 in an African setting)
- At least 2 sites that have problems to cope with current workload

#### Population Source

Sputum samples from all known or suspected TB patients which are submitted for routine microscopic examination during the demonstration project period will be included in the study.

#### Partners

- **India** (9 demonstration and 3 supervisory sites: Christian Medical College Vellore, JALMA Agra and New Delhi TB Centre)
- **Vietnam** (3 demonstration and 1 supervisory site: Pham Ngoc Thach Hospital, Ho Chi Minh City; coordinated by KNCV)
- **Peru** (3 demonstration sites and 1 supervisory site: Instituto Nacional de Salud, Lima)
- **Russia** (2 demonstration sites and 1 supervisory site: District Laboratory, Samara)
- **Lesotho** (2 demonstration sites and 1 supervisory site: National Reference Laboratory, Maseru)
- **Ethiopia** (3 demonstration sites and 1 supervisory site: EHNRI National Reference Laboratory, Addis Ababa)
- **Tanzania** (2 demonstration sites and 1 supervisory site: Muhimbili National Reference Laboratory, Dar es Salaam)
- **Cambodia** (2 demonstration sites and 1 supervisory site: National TB Reference Laboratory, Phnom Penh)
- **Thailand** (2 demonstration sites and 1 supervisory site: National TB Reference Laboratory, Bangkok)
- **Other countries:** 20 microscopes will be provided to FIND partners including IUATLD, CDC, PATH, and BMGF to expand the study to other countries and/or answer specific questions.

#### Study Design

The Primo Star iLED will be implemented in at least 20 routine microscopy centers without prior experience in fluorescence microscopy to determine operational and clinical performance in the intended settings of use as well as acceptability for the laboratory staff and cost-effectiveness. Participating microscopy centers will be grouped in clusters. Each cluster will consist of one supervisory site and two to three microscopy centers. The

supervisory site will be responsible for training, monitoring, rechecking of slides and data management.

### Study Hypothesis

We postulate that the Primo Star iLED system is a feasible, advantageous and cost-effective replacement for ZN (and, where existing, conventional fluorescence) microscopy in low- to moderate-income laboratory settings. Especially in busy microscopy centers, it will increase the case detection rate while substantially decreasing the daily workload.

### Endpoints

FIND believes that large-scale demonstration projects are required to provide the evidence that diagnostic tests performing well in affluent countries can successfully be implemented and have significant medical and public health impacts in programmatic settings. The purpose of this demonstration project is to assess the implementation of Primo Star iLED as replacement for ZN in routine TB diagnosis in low- and moderate-income settings. Specifically, we are interested in the following:

#### Primary Objectives (*Basic Protocol*)

1. To assess the feasibility of implementing Primo Star iLED for TB diagnosis at microscopy centers without prior experience with fluorescence microscopy in low- to moderate-income settings and to identify barriers to implementation
2. To determine the false positivity and negativity rate of LED fluorescence reading compared to a ZN baseline and to results from the supervisory site
3. To determine the development of false positivity and negativity rates of LED fluorescence reading over time (with increasing experience)
4. To assess the impact of this implementation on daily workload and case detection rates for low, middle and high-volume settings
5. Determine lab technicians' appraisal of Primo Star iLED
6. To evaluate detailed costs associated with LED-based fluorescence microscopy in comparison with conventional methods

#### Secondary Objectives (*Extended protocol - FIND partners*)

1. In collaboration with other groups and in preparation for WHO STAG submission, establish comparative performance data for alternative LED-based approaches
2. To identify minimal training needs and develop training modules accordingly
3. To identify the optimal fluorescence staining method and ensure continuous and affordable supply
4. To assess effects of fading speed on external quality assurance by rechecking
5. To assess whether combining LED fluorescence microscopy with concentration methods such as bleach sedimentation further increases sensitivity

## Study phases

### *a) Investigators' meeting (duration: 1 day)*

An investigators' meeting was held in March 2008 to refine the master protocol and get input from national TB programs and other FIND partners, adapt questions to country-specific situations, identify supervisory sites and participating microscopy centers and to develop detailed, country-specific study plans.

### *b) Establishing ZN baseline (duration: 1 month)*

In order to be able to compare false positivity and negativity rates for LED fluorescence with the respective rates for ZN reading during the implementation phase, it will be necessary to establish a baseline under study conditions. Using data available from the routine quality assurance program would possibly result in a bias, since performance is likely to be better under study conditions simply due to the fact that staff get more attention than usual and are therefore more motivated. As a result, participating laboratory technicians will receive instructions for the study and study-related data management prior to this phase. All incoming sputum samples will be examined by ZN microscopy according to program norms and kept in slide boxes provided by FIND. All slides will be rechecked by the supervisory site and discrepant cases resolved by the Supra National Reference Center in Germany.

### *c) Training (duration: 5 days)*

The supervisory site(s) in each country (supported by FIND and its partner institutions) will be responsible for training the laboratory staff from participating microscopy centers. This training shall take approx. 5 days, but can be shorter for staff with prior experience in fluorescence microscopy. An important outcome of this demonstration study shall be to help define minimal training needs and best training modules. FIND will provide training materials.

### *d) Validation (duration: 1 month)*

The training will be followed by a validation phase of, during which an Auramine slide will be prepared for all incoming sputum samples and will be examined by Primo Star iLED using 40x magnification. On a daily basis, all slides will be rechecked by the supervisory site using conventional fluorescence microscopy. Results will be provided to the microscopy center the following day, and treatment will be started on the basis of the conventional fluorescence results. At the end of this validation, a proficiency slide panel of 10 ZN and 10 Auramine slides will be provided to all participating sites.

Performance targets for validation phase and proficiency panel:

- *>95% accordance between results of the demonstration site and supervisory site for fluorescence readings*
- *Quality of Auramine stains acceptable in 100% of panel slides examined*
- *≤ 2 false results in the proficiency testing panel*

Sites that meet these performance targets are ready to enter the implementation phase. Sites that fail to meet performance targets will receive additional training and undergo proficiency testing until targets are met.

*e) Implementation phase (duration: 3 months)*

After a successful training and validation phase, all incoming sputum samples will undergo routine microscopic examination with the Primo Star iLED according to program norms, and patients will be managed based on Primo Star iLED results for a period of 3 months. All slides will be stored in slide boxes for rechecking. Intensive rechecking of up to 100% of slides will take place at the respective supervisory laboratory: This will allow determining the false positivity / false negativity rate over time for each demonstration site. However, in order to be able to compare these rates to ZN, it will be necessary to establish a false positivity / false negativity baseline for ZN beforehand. Rechecking will be performed by experienced staff only and all discrepant slides (slides for which the result of the microscopy center is positive but the one from the rechecking supervisory site negative, or the other way round) will be reexamined by the Supra National Reference Laboratory. Prevalence data during implementation will be compared with preceding quarters. Laboratory technicians' appraisal of the new method will be assessed both initially and after 3 months with the help of a questionnaire. In addition, cost data for LED fluorescence, compared to ZN and standard fluorescence microscopy, will be collected for selected sites and countries. Supervisors will visit microscopy centers once every second week to discuss and record any problems with staining solutions, the microscope or result interpretation.

*f) Continuation and expansion phase (duration: 6 months)*

In collaboration with other international partner organizations and national TB programs, the demonstration project will be expanded to other countries/sites. In general, rechecking of slides, supervision and monitoring will be reduced compared to the implementation phase. Rechecking will be done according to national TB guidelines. Specific operational issues with respect to fluorescence microscopy will also be addressed during this phase. For example:

- Identify optimal fluorescence staining method (methylene blue or pelican ink are possible counterstains that may produce a lighter background and therefore allow easier focus; phenol free staining kits have become available on the market and may be considered as environment-friendly alternatives)
- Determine effect of fading speed on external quality assurance by rechecking
- Ascertain effect of combining LED fluorescence microscopy with methods such as bleach sedimentation to further increase sensitivity
- Determine performance specifically in high HIV prevalence settings and overburdened microscopy centers

### Study Design Overview

Study phase	Duration	% slides re-checked	Staining reagents	Microscope for reading	Microscope for re-checking	Patient management	Frequency of retrieving slides /forms	Supervisory visits with checklist	Forms	Data transfer by courier
<b>ZN Baseline</b>	1 month	100%	Routine Zn stain	Conventional Brightfield (1000X)	Conventional Brightfield (1000X)	Based on ZN result of microscopy center	Once every 2 <sup>nd</sup> week	Monthly	1. Result Form: ZN Baseline 2. Rechecking Form: ZN Baseline	At the end of phase
<b>Training</b>	5 days									
<b>Proficiency testing &amp; User appraisal</b>	1 day	100%	For 10 Au and 10 ZN slides	Primo Star iLED (400X) Conventional Brightfield (1000X)	Only for discrepant: Primo Star iLED (400X) Conventional Brightfield (1000X)	-	-	-	1. Proficiency Testing Result Form: 2. User appraisal questionnaire	Scanned by e-mail following day
<b>Validation</b>	Minimum 1 month. Until targets met.	100%	Au staining reagents provided by supervisory site once per month	Primo Star iLED (400X)	Conventional FM (200-250X) (where not available Brightfield after restaining (1000X))	Based on conventional FM result from supervisory site (Brightlight if not available) <i>!Daily provision of results!</i>	Daily	Every 2 <sup>nd</sup> week	1. Result Form: Validation 2. Rechecking Form: Validation	Every 2 <sup>nd</sup> week
<b>Proficiency testing &amp; User appraisal</b>	See above									
<b>Implementation</b>	3 months	As per LQAS	Au staining reagents provided by supervisory site once per month	Primo Star iLED (400X)	Primo Star iLED (400X)	Based on iLED result from microscopy center	Once every 2 <sup>nd</sup> week.	Monthly	1. Result Form: Implementation 2. Rechecking Form: Implementation	Monthly
<b>Proficiency testing &amp; User appraisal</b>	See above									
<b>Continuation</b>	6 months	As per NTP	Au staining reagents by supervisory site	Primo Star iLED (400X)	Primo Star iLED (400X)	Based on iLED result from microscopy center	Monthly	Monthly	Same as implementation	Monthly

### Ethical considerations

Patients will be treated on the basis of the Primo Star iLED result, which implies replacing the currently used routine ZN result. Ethical approval will therefore be required for all participating sites/countries. Risks: Numerous published studies have demonstrated that fluorescence microscopy is more sensitive than and equally specific as ZN microscopy. Fluorescence microscopy is a well established method for TB diagnosis, particularly in high-income countries. Thus, participation in this project poses no foreseeable risk to patients. No informed consent will be sought for this demonstration project and a waiver of informed consent will be requested from relevant Institutional Review Boards (IRBs). All patients will receive diagnosis and treatment according to program norms throughout the demonstration period. Confidentiality: There are no risks with respect to confidentiality. No personal health information or demographic data will be collected for this laboratory study.

### Data management and potential use of findings

All forms required for data entry will be provided by FIND. The electronic data entry tool for the study will be connected to FIND's central database through secured VPN, which offers the advantage that study monitors will have continuous access to the electronic data. Electronic data entry will be conducted by professional staff hired in India. This data entry staff will receive source data from the supervisory sites every 2-4 weeks. The original forms must be stored on site for at least 3 years after study completion.

Toward the end of the project, basic protocol data will be pooled, analyzed, and the results disseminated by FIND in collaboration with all project sites. The data will be reported to local and national TB and public health authorities such as the WHO Strategic Technical Advisory Board to inform policy decision. Additionally, the findings of this project will be published in a peer-reviewed journal and may be used to encourage other TB programs to evaluate and implement LED-based fluorescence microscopy. In its charter, FIND states that it is responsible for ensuring such dissemination. Furthermore, FIND will compile and analyze the data from all the sites and prepare a first draft for review by the sites. A final publication will be prepared with input and authorship from all the collaborators. Site-specific data may be presented independently and in combination with other data, but only after the principal publication has been made available and/or accepted for publication. In the case where the combined multi-site data are not of publishable quality, FIND shall be the final arbiter on any joint publication. All scientific manuscripts and reports related to these studies should be sent to FIND for review prior to submission for publication.

### Standardization and Quality Control (QC)

Staining solutions will be prepared by supervisory sites using reagents from Merck based on a SOP provided by FIND. Incoming QC and lot-to-lot QC will be performed according to this SOP. Data will be captured on laboratory forms provided by FIND, which will then be sent to a central data entry unit in order to be captured electronically. Semiquantitative

results for ZN will follow the scale used by the local NTP and for fluorescence will follow the IUATLD/WHO scale. All slides will be stored on site according to an SOP. Rechecking will be standardized for all participating countries. In brief, highly experienced microscopists at the supervisory sites will read 300 fields per slide for negative smears, 100 fields for scanty smears until a semiquantitative result has been established for higher positive smears. Slides with discrepant results will be rechecked by the Supra National Laboratory in Germany using LED microscopy and assessing the same number of fields. Their conclusions will overrule the results from the microscopy center and demonstration site.

IUATLD/WHO SCALE (1000x field=HPF)  Result	MICROSCOPY SYSTEM USED		
	BRIGHTFIELD (1000x magnification; 1 length = 2cm = 100 HPF	FLUORESCENCE (200-250x magnification; 1 length = 30 fields = 300 HPF	FLUORESCENCE (400x magnification; 1 length = 40 fields = 200 HPF
<b>Negative</b>	Zero AFB/1 length	Zero AFB/1 length	Zero AFB/1 length
<b>Scanty (actual count)</b>	1-9 AFB/1 length or 100 HPF	1-29 AFB/1 length	1-19 AFB/1 length
<b>1+</b>	10-99 AFB/1 length or 100 HPF (=1-9 AFB/10 fields)	30-299 AFB/1 length	20-199 AFB/1 length
<b>2+</b>	1-10 AFB/1 HPF on average	10-100 AFB/1 field on average	5-50 AFB/1 field on average
<b>3+</b>	≥10 AFB/1 HPF on average	≥100 AFB/1 field on average	>50 AFB/1 field on average

Table 5: Semi-quantitative scale

### **Blinding**

Blinding at the microscopy centers will not be required. The slides will be labeled according to routine procedures. During the implementation phase, rechecking at the supervisory site will be done without access to results from the microscopy centers.

### **References**

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