# Harmonizing the Use of Molecular & Culture-based DST of *Mycobacterium tuberculosis*

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# Harmonizing?

- There must be some conflicts?
- Yes!
- Nothing is perfect, but each has its strength!
- How to make best of both methods to achieve the goal of providing timely, accurate DST results to clinicians—that is the work of harmonizing!

# Disclaimer

- No financial affiliation with the companies whose products will be discussed in this presentation.

# Acronyms

- DST: Drug susceptibility testing
- MDST: Molecular DST
- CDST: Culture-based DST
- AP: agar proportion method
- DR/DS: drug resistance/susceptibility
- SQ: sequencing or sequence
- TAT: turnaround time

# What are not in “harmony”?

- MDST detected no mutations; CDST tested R.
- MDST detected a mutation; CDST tested S.
- Discrepancies occur:
  - between CDST methods: AP & MGIT disagree
  - between labs or within lab, by different methods or even the same method.
- Discrepancies between MDST methods.
- Which result to trust? Headaches for clinicians!

# Comparison

| | MDST | CDST |
|---|---|---|
| TAT | Fast (1-3 days) | Slow (weeks) |
| Availability | Limited drugs | Most drugs |
| Requires pure and viable cultures | No | Yes |
| Sensitivity & Specificity | Not 100% | |

# MDST--benefits

- Quick screening for DR
  - No mutations detected, it provides good prediction for DS and confidence for continuation of standard regimen.
- Provides quick results for mixed or contaminated cultures, or negative cultures if smear-positive sediments are available.
- Provides quick confirmation of DR found by CDST.
  - Rule in DR, if mutations detected.

# CDST—we still need it!

- CDST results, if correct, overrides MDST results
  - Sensitivity of MDST is not 100%
    - MDST detected no mutations, CDST can still be R.
      - Need to verify CDST results are correct—if in doubt, re-test making sure culture is pure & DST is properly performed.
  - Specificity of MDST is not 100%
    - MDST detected mutations but CDST can be S.
    - Need to recognize those mutations—silent mutations & others
      - Some MDST methods do not have this capability.
  - Association of some mutations with DR is not certain.
    - Need to rely on CDST. [more to discuss]

# MDST methods

- Probe-based
  - Molecular beacons (GeneXpert)
    - Xpert got FDA-approval last month!
  - Line-probes (HAIN & LIPA)
- SQ-based
  - Sanger sequencing
  - Pyrosequencing

# What to expect from various MDST methods

- Molecular beacons—
  - Mutation present or absent.
- Line-probes—
  - Few bands with specific mutations.
  - Presence or absence of wildtype bands.
- SQ methods—
  - Provide SQ.
  - Allow to identify specific mutations—known & new.

# GeneXpert (GX)

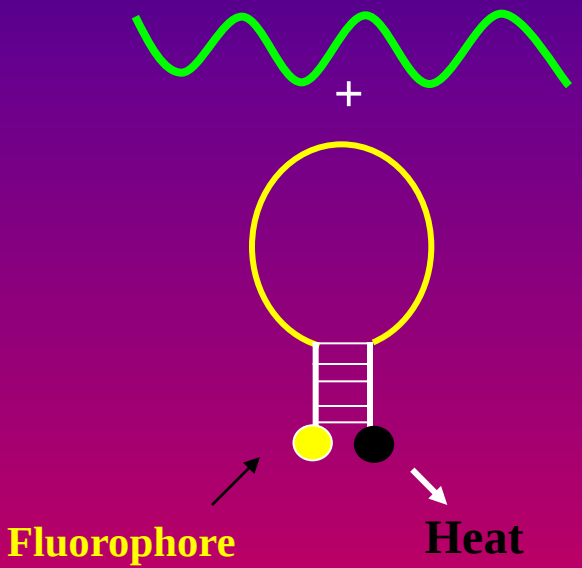
- For identification of MTB & detection of RIF-R
- Simple, fast—hand-on time: few minutes.
- Testing raw specimens in 2 hr.
- Performance—
  - Identification of MTBC—
    - Sensitivity: Smear +: 98.2%; Smear-: 72.5%
    - Specificity: 99.2%
  - Detection of RIF-R
    - Sensitivity: 97.6%
    - Specificity: 98.1%

- Reference: Rapid molecular detection of TB & RIF resistance. NEJM 2010;363:1005-1015. Boehme et al.
- Be aware: there are limitations!

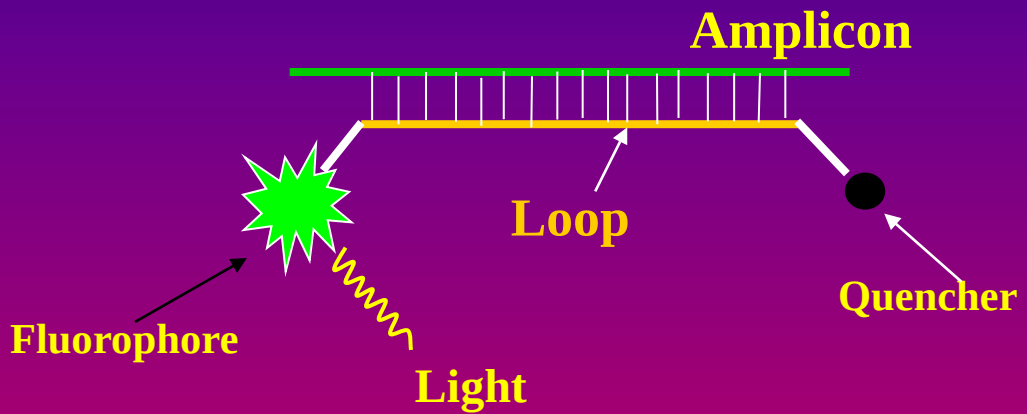
# Detection of Mutations with a Molecular Beacon

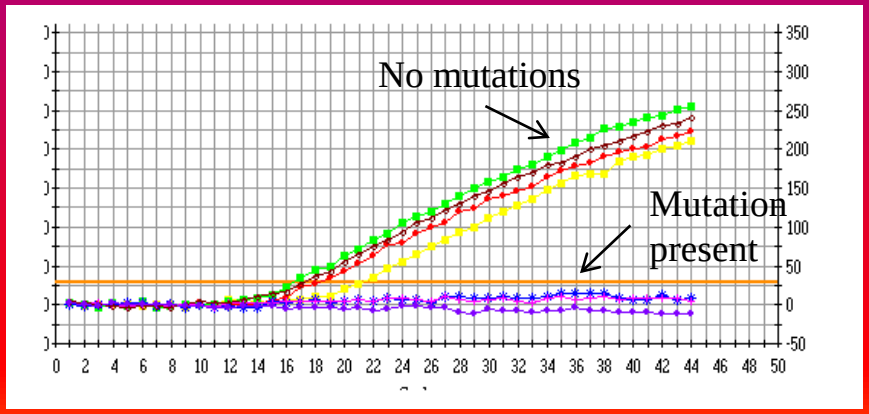
## (Loop portion containing wildtype SQ)

**Mutant Sequence**




**Wildtype Sequence**

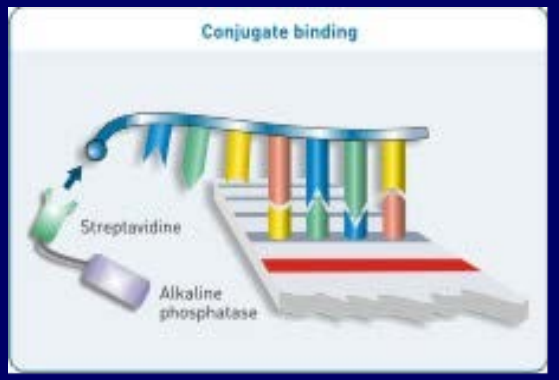


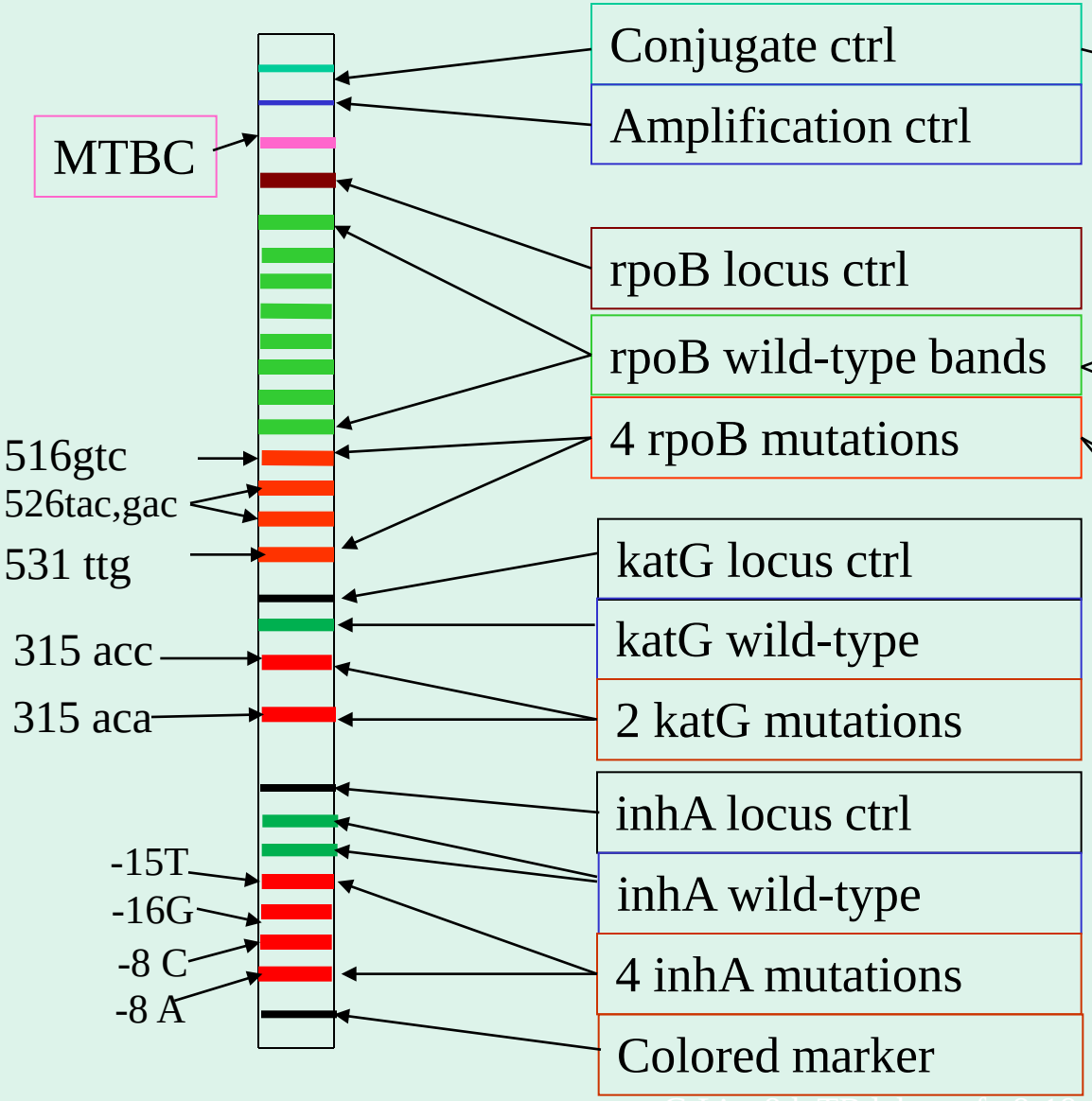

Courtesy of Dr. Probert

# Line Probe Assays

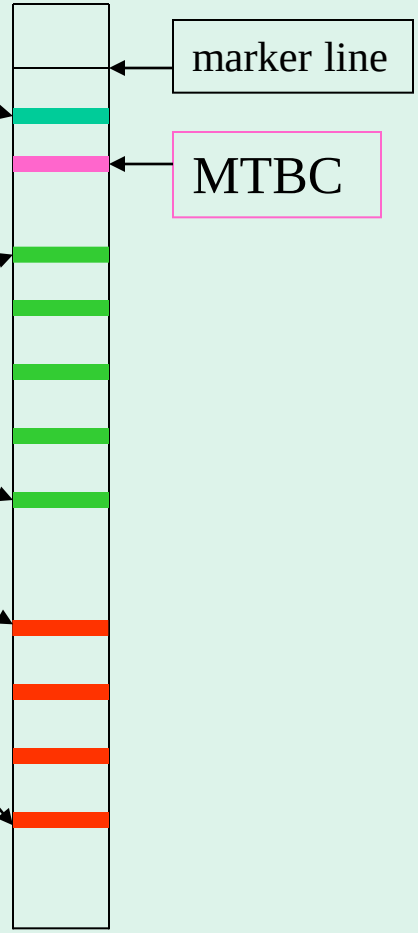
- Amplification of the target
  - Traditional PCR; one primer is biotinylated.
- Reverse hybridization
  - ssDNA-biotin hybridize to probes on membrane, if complementary.
  - Colorimetric detection
    - Observe presence or absence of bands.



# MTBDRplus by HAIN

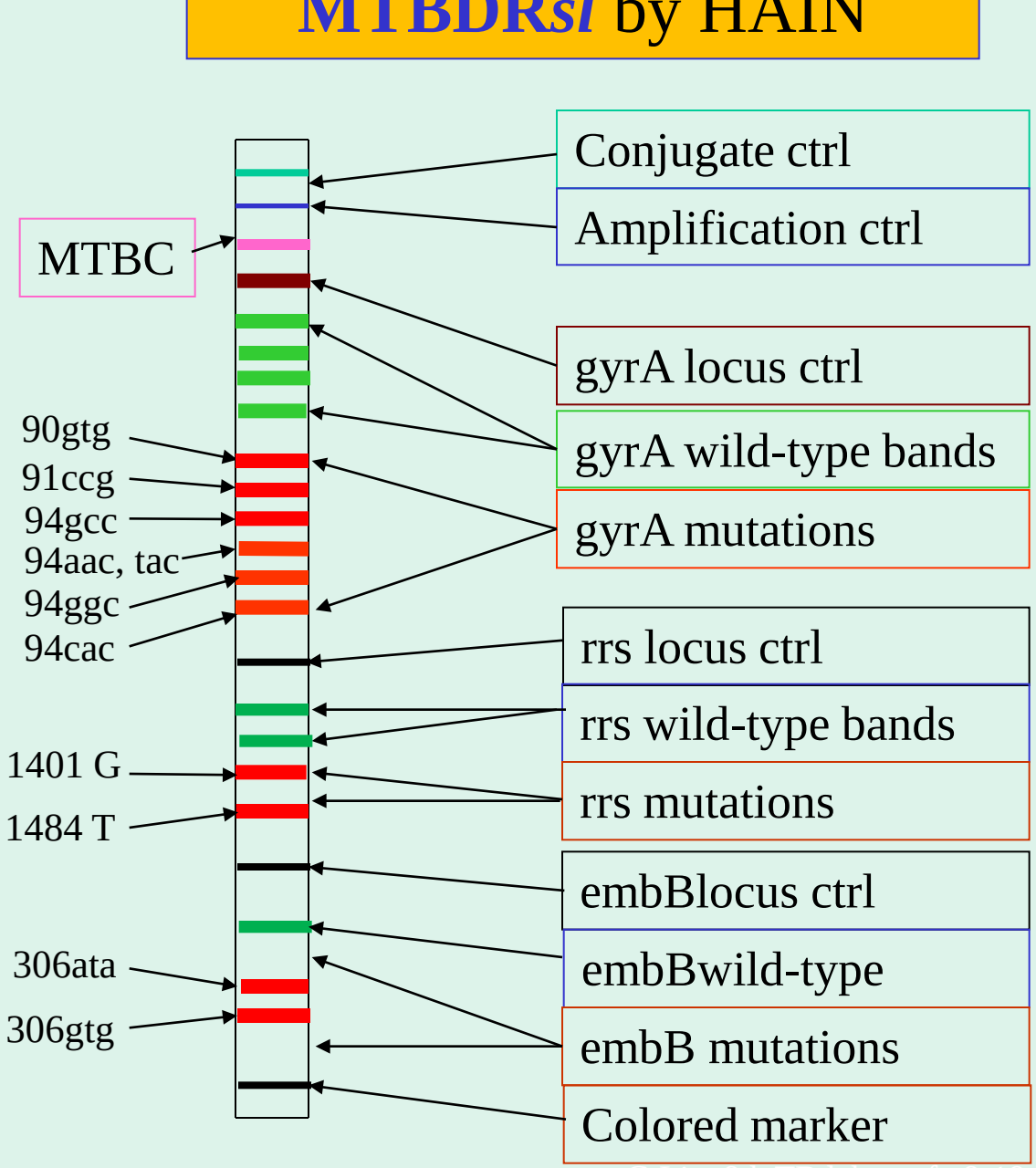


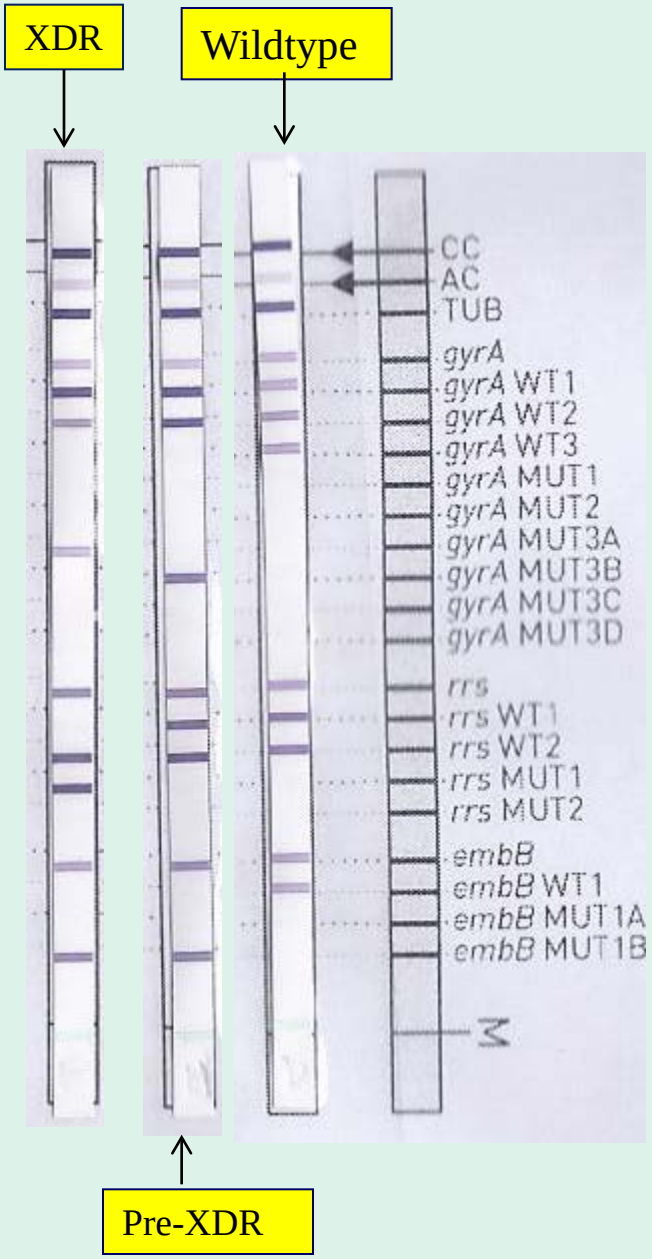

# LiPA RIF.TB by INNOGENETICS




Detect presence or absence of mutations & a few specific mutations.

# MTBDR*sl* by HAIN



# Probe-based MDST

- Detect presence or absence of a mutation(s).
- Do not inform what the mutation is
  - Assume all mutations are associated with DR.
  - Often true, but not always.
- When RIF-R rate is 2%, if the specificity is 96%, the PPV is 33%. [CA data]
- Due to low PPV, when a mutation is detected, confirmation by a sequence-based method is recommended.

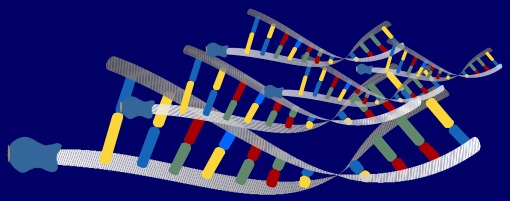
# GeneXpert

- No mutations detected in *rpoB*
  - Predict RIF-S, but unable to predict for INH
  - If INH-R is suspected, use other MDST methods
    - HAIN, PSQ or Sanger
- Mutations detected in *rpoB*
  - If RIF- R is not suspected, confirmation by a SQ-based MDST is recommended.
  - If RIF- R is suspected, confirmation by a SQ-based MDST is optional.
    - Probe E—most common, likely be true +

# Sequence-based MDST

- Sanger sequencing
  - Gold standard http://en.wikipedia.org/wiki/Sanger_sequencing
- Pyrosequencing (PSQ)
  - Realtime sequencing
  - http://www.qiagen.com/media/player.aspx?movie=Pyrosequencing&width=480&height=380

# Pyrosequencing workflow



1-2h

30-50 min

1 min/base

**PCR**

**ssDNA prep**

**PSQ**

Amplify target.

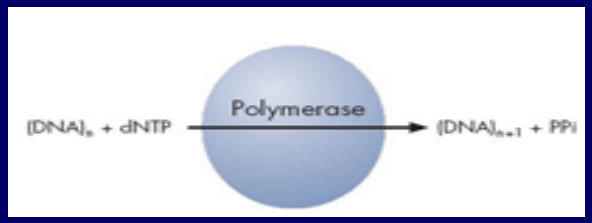
One primer is biotinylated.

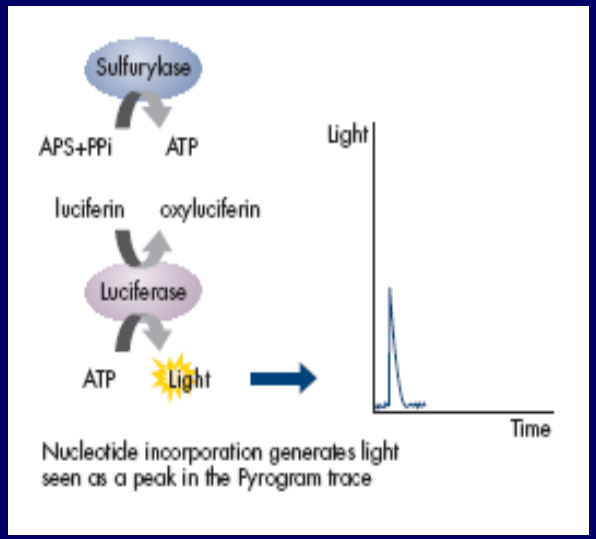
Capture ssDNA with streptavidin-coated beads.

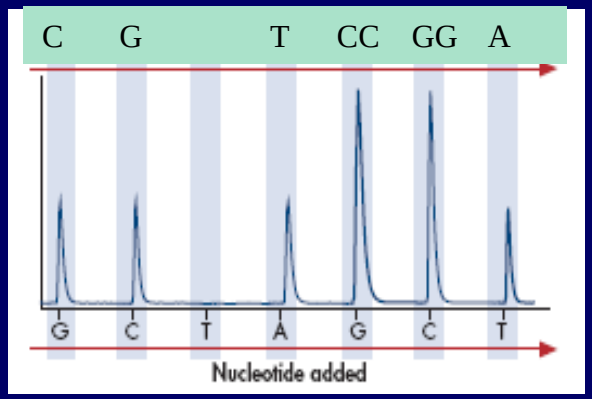
SQ primer anneals to DNA.

Realtime sequencing.

SQ data generated from the 1st base next to SQ primer.



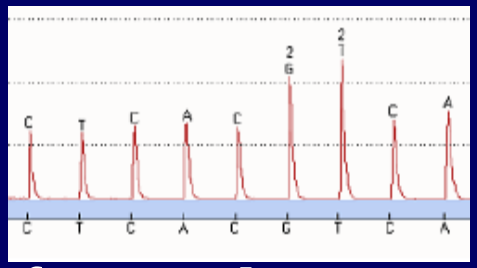



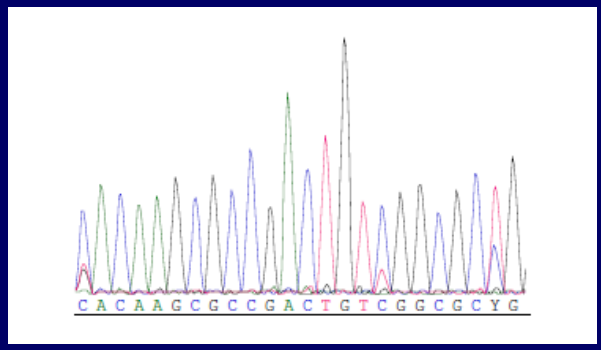

## Below are steps occur in pyrosequencer:

1. Incorporation of dNTP generates ppi.
2. APS + ppi ⟶ ATP, catalyzed by ATP sulfurylase.
3. ATP drives Luciferin ⟶ oxyluciferin, catalyzed by luciferase.
4. Light generated, proportional to dNTP incorporated, recoded by CCD.
5. Apyrase degrades unincorporated dNTP & ATP. When degradation is complete, another dNTP will be added.
6. Pyrogram shows sequential event of dNTP incorporated. The peak level is proportional to dNTPs incorporated.

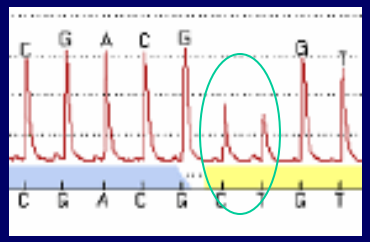
# SQ-based MDST



- Transparency with SQ provided
  - Enable to differentiate silent mutations from others.
  - Prevent reporting false-R.



- New mutations can be detected.
  - Defer interpretation to CDST.
- Emerging resistance can be recognized to certain extent (5:5 or 4:6, etc.).



- Allow to study MIC for each mutation
  - This knowledge is accumulable for future use.

# SQ-based MDST

- No mutations detected –predicts DS.
- Mutations detected
  - For known mutations, based on past experience to interpret.
    - All mutations are not equivalent, some may not confer DR.
    - It is advisable to study MIC for each mutation.
  - Silent mutations are not associated with DR.
  - If the association of a mutation with DR is uncertain, defer interpretation to CDST.
  - For new mutations, defer interpretation to CDST.

# Recommendations--CDST

- Two scenarios:
  - If DR detected, but not anticipated
  - Discordant CDST results with different methods or same method.
- Performing MDST can be helpful
  - If mutation detected, confirms DR results.
  - If mutation not detected, lean toward DS, repeat CDST.
    - Make sure the culture is pure
    - DST is performed properly
      - Drug solution is prepared and added properly
      - Inoculum is prepared properly.

# Discordant CDST results: hard to resolve

- Sometimes even MDST cannot help!
  - Association of some mutations with DR is uncertain!
    - *embB* mutations—may not confer EMB-R
    - *rrs* 1401A/G– msy not confer CAP-R
    - Association of many *pncA* mutations with PZA-R is still unknown.
- Problems with test (critical) concentrations?
- Problems with CDST methods?

# Discordant CDST results "Flip-floppers"

- Poor reproducibility of CDST may occur when:
  - Some mutations cause MIC elevated slightly but lower than or close to the critical concentration.
  - Some strains with wildtype SQ (or have a mutation somewhere we do not know), but MIC of a drug is close to the critical concentration.

# Flip-floppers (examples)

- rpoB 533 CCG & 511CCG
  - Borderline S or low level R.
  - RIF MIC = 0.5 ug/ml by MGIT
    - MGIT: likely to be S.
  - AP: S or R (low % R)
- rrs 1402C/T—AMK-R or S
  - Borderline S or low level R.

# Flip-floppers = Borderline??

- TB DST does not have borderline category
  - Exception: PZA testing by BACTEC 460.
- For isolates with flip-flopping results
  - Lab may repeat and repeat, then report R or S based on 2/3 or 3/5 winners.
  - Clinicians takes S or R without knowing the flip-flopping nature of the result.
- Is it advisable to consider “borderline” for those flip-floppers?
  - When arsenals fall short for difficult TB cases, those flip-floppers may still be useful?

- A lot more to research.
- Microbes are smart!
- But, we do not give up!!

# Thank You!

# Comments & Questions!