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New Tools to Manage an Old Disease – Genotyping and QuantiFERON

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Genotyping - Definition

- The molecular characterization and utilization of nucleic acid regions or elements within *M. tuberculosis* genome for identification purposes.
- AKA - Fingerprinting

Nucleic Acid – Based Genotyping Methods

1) Restriction Fragment Length Polymorphism (RFLP) analysis

- Utilize well-characterized repetitive element Insertion Sequence 6110 (IS*6110*)
- Standardized methodology - van Embden
- Computerized laboratory database

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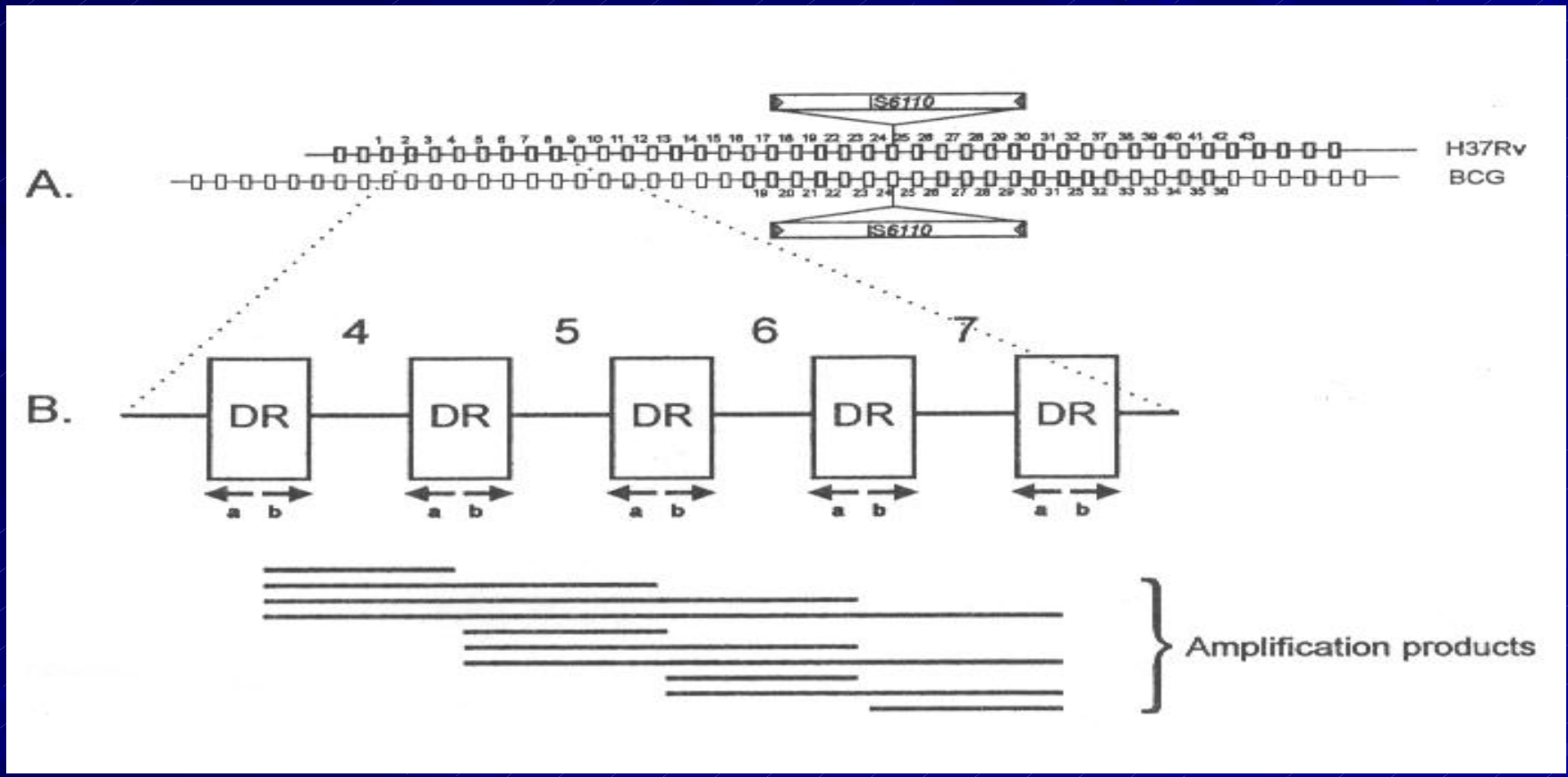
Nucleic Acid – Based Genotyping Methods (2)

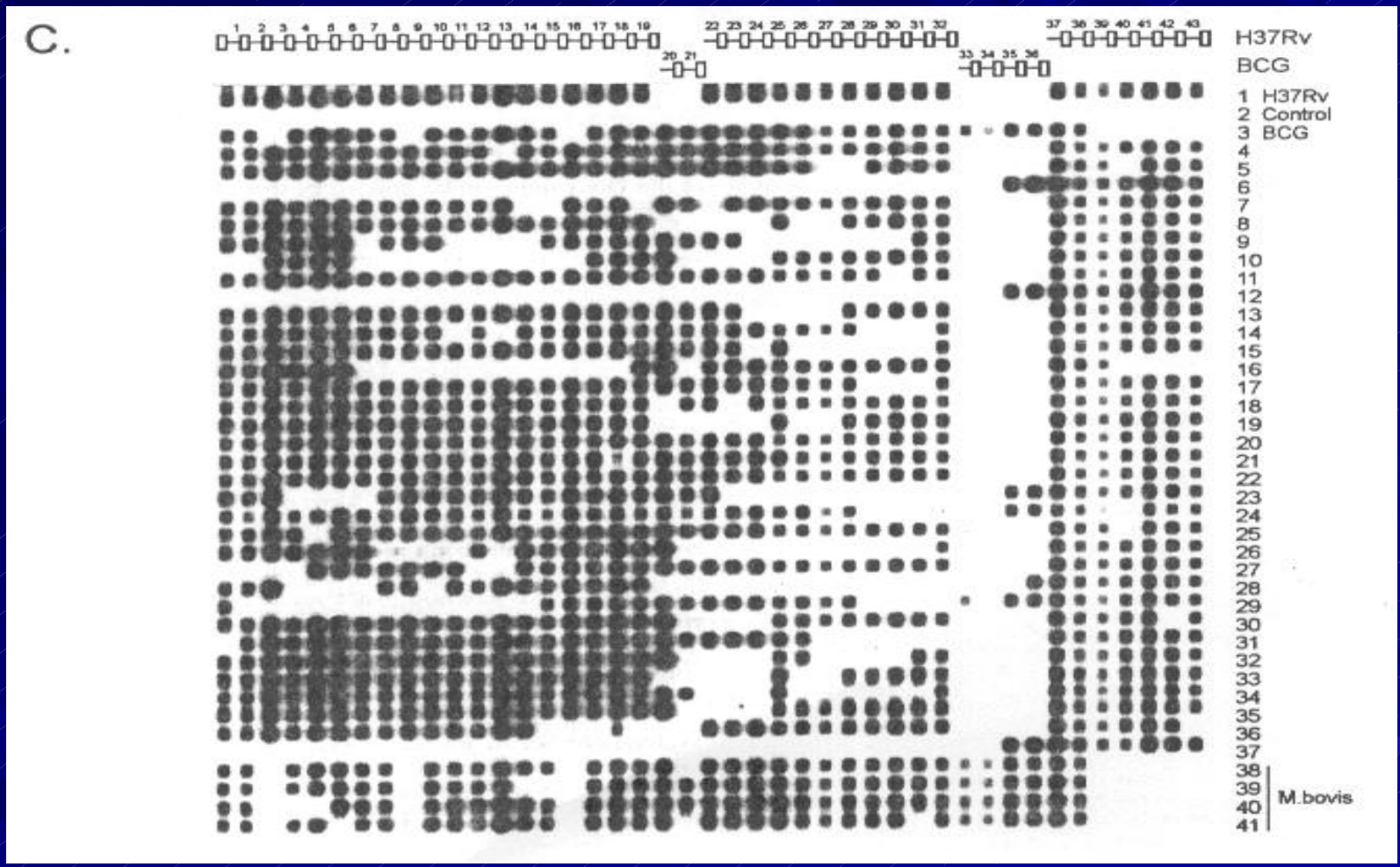
2) Spoligotyping - Spacer oligonucleotide typing

- PCR method exploiting DNA polymorphisms within the direct repeat (DR) locus of MTB
- Locus contains multiple, well-conserved 36 bp DRs interspersed with non-repetitive spacer sequences 34-41 bp long
- Variation in the 43 spacers reflects the polymorphisms studied

Spoligotyping Schematic



Spoligotyping Schematic



Spoligotyping Advantages

a) Smaller amounts of DNA needed, so procedure can be run on clinical samples or on strains shortly after liquid inoculation

b) Results expressed in digital format –

7777 7677 7760 771 Print 4

7777 7747 7760 631 H37Rv

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Nucleic Acid – Based Genotyping Methods (3)

3) MIRU-VNTR

- PCR method characterizing number and size of variable number of tandem repeats in each of 12 independent mycobacterial interspersed repetitive units
- Appropriate for all MTB strains no matter the IS*6110* number
- Rapid comparison of results (12 digit classification system)

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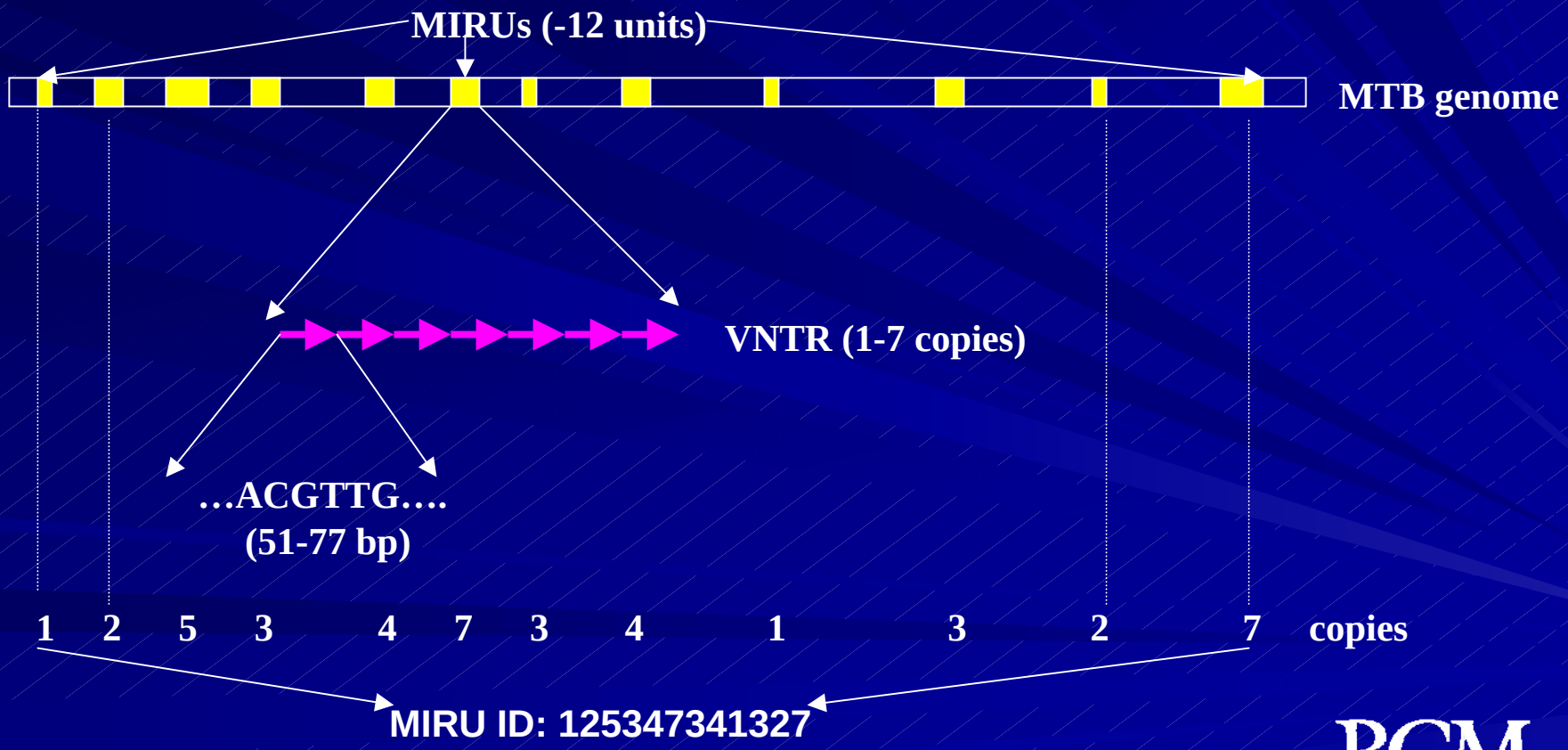
MIRU-VNTR Schematic



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Genotyping Assumptions

- Epidemiologically related strains will have the same genotype pattern and unrelated strains will have different patterns.
  - Same patterns = cluster = ongoing transmission
  - Different patterns = unique, non-clustered = reactivation or LTBI

Lessons Learned (1)

- Infectiousness of patients – "bar-hoppers"
- Recurrent and exogenous reinfection - In Houston relapse of new strain (24-31%)
- Impact of drug resistance on transmission – drug-resistant strains less likely to be clustered

Lessons Learned (2)

- Contact and outbreak investigations – transmission can occur through short-term, casual contact; ineffective contact tracing
- Measure the performance of TB control programs – proportion of cases that are clustered
- Geographic distribution and dissemination of MTB – 25% of clustered isolates in Houston are Beijing-family strains

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QuantiFERON

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QuantiFERON

- Until recently, the TST was the only method available for diagnosis of LTBI
- Utility of TST hampered by:
  - Potential for false positive and false negative results
  - Administration and interpretation
  - Difficulty in separating true infection from the effects of prior BCG vaccination
  - Infections caused by non-tuberculosis mycobacterium (NTM)

QuantiFERON

- Advances in genomics and immunology have led to a promising alternative – *in vitro* interferon-gamma (INF☎) assays

- **Principle** – T-cells of individuals infected with *M. tuberculosis* release INF☎ when they re-encounter TB-specific antigens.

QuantiFERON Antigens

- **QuantiFERON** (first generation) – Similar to PPD at least 100 different mycobacterial antigens
- **QuantiFERON – Gold** (second generation, 2G) – Use two mycobacterial specific antigens: early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP10)
- **QuantiFERON – Gold *In Tube*** (third generation, 3G) – ESAT-6; CFP10; and TB7.7 (Rv2654)

Advantages of INF☎ Assays

- Higher specificity – less influence by BCG vaccination
- At least as sensitive as TST in active TB
- Need for fewer patient visits
- Avoid subjective readings
- Ability to perform serial testing without boosting

Limitations of QuantiFERON

- Higher costs -

  TST = $ 9.79 (Center for Medical Services, CPT 86580)

  QFT-Gold = $37.39 (BMC Infectious Diseases 2006, 6:47)

- Need for laboratory support –

  Courier to laboratory for processing on the same day as phlebotomy (12 hour window)

- Need for venous blood –

  Heparinized whole-blood assay

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Limitations of QuantiFERON

- Package Insert – “Gold not evaluated for use in”
  - Immunosuppressed
  - < 17 years of age
  - Pregnant women
- Lack of published data

Stage 1 (QuantiFERON – Gold)

- Aliquot 1ml of heparinized whole blood into 4 wells of a 24/96-well culture plate
- Add 3 drops of antigens/controls

FIGURE 1. Recommended layout for dispensing Blood and Stimulation Antigens into 24 Well Culture Plates

	Patient Sample Number 1	2	3	4	5	6
Nil Control *(gray cap)*	◯	◯	◯	◯	◯	◯
ESAT-6 *(red cap)*	◯	◯	◯	◯	◯	◯
CFP-10 *(white cap)*	◯	◯	◯	◯	◯	◯
Mitogen *(purple cap)*	◯	◯	◯	◯	◯	◯

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Stage 1 - continued

- Shake covered plate for 1-2 minutes
- Incubate at 37°C (humidified) for 16-24 hours
- Harvest ≥ 200ml plasma from each well
- Store plasma in racked microtubes or uncoated microtitre plate:

2 – 8°C	< 28 days
-20°C	Up to 3 months

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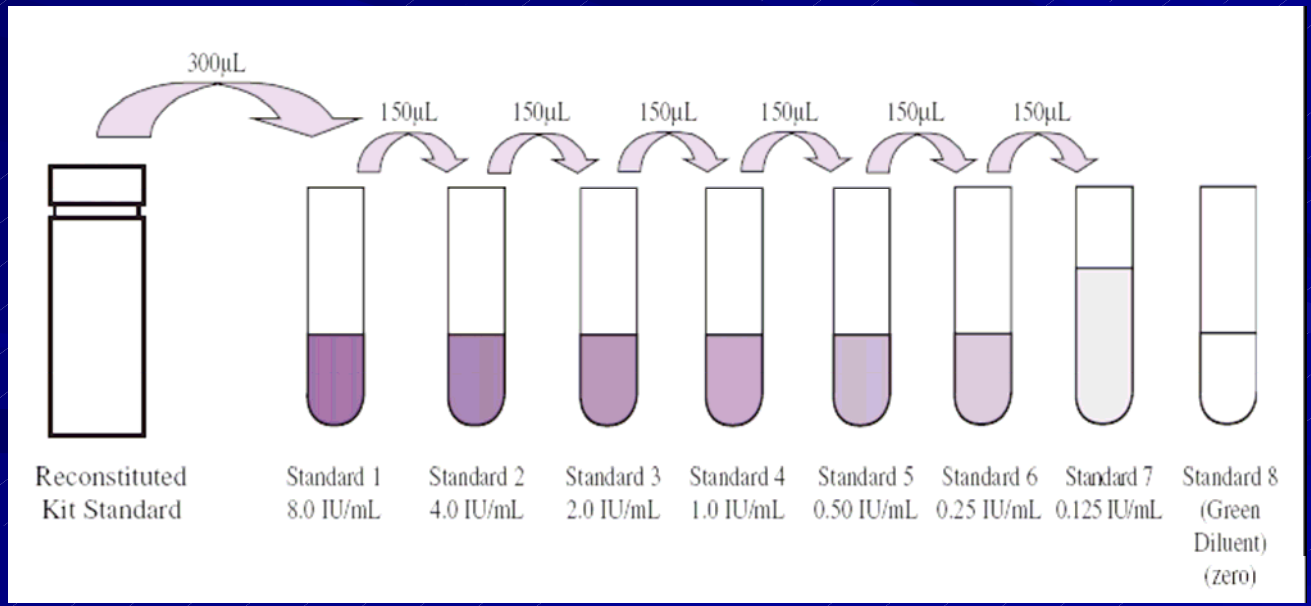
Stage 2 and Standard Preparation

- Add 50ml of conjugate solution to each well
- Add 50ml plasma/standard to the appropriate wells



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96-well format

FIGURE 2. Recommended Sample Layout - Whole Plate

Row	1	2	3	4	5	6	7	8	9	10	11	12
A	1N	2N	3N	4N	5N	S1	S1	6N	7N	8N	9N	10N
B	1E	2E	3E	4E	5E	S2	S2	6E	7E	8E	9E	10E
C	1C	2C	3C	4C	5C	S3	S3	6C	7C	8C	9C	10C
D	1M	2M	3M	4M	5M	S4	S4	6M	7M	8M	9M	10M
E	11N	12N	13N	14N	15N	S5	S5	16N	17N	18N	19N	20N
F	11E	12E	13E	14E	15E	S6	S6	16E	17E	18E	19E	20E
G	11C	12C	13C	14C	15C	S7	S7	16C	17C	18C	19C	20C
H	11M	12M	13M	14M	15M	S8	S8	16M	17M	18M	19M	20M

S1–8 (S1: Standard 1, S2: Standard 2, S3: Standard 3, S4: Standard 4, S5: Standard 5, S6: Standard 6, S7: Standard 7, S8: Standard 8); 1N (Sample 1 Nil Control plasma); 1E (Sample 1 ESAT-6 plasma); 1C (Sample 1 CFP-10 plasma); 1M (Sample 1 Mitogen Control plasma)

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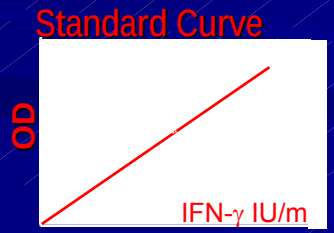
Stage 2 - continued

- Shake covered plate for 1 minute
- Incubate at room temperature for 2 hours
- Wash plate ≥ 6 times
- Add 100ml of substrate
- Incubate for 30 minutes at room temperature
- Add 100ml of stop solution
- Read absorbance at 450nm within 5 mins



Measure OD and determine IFN-γ levels

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Results Interpretation

Mitogen-Nil[1] IU/mL	ESAT-6 - Nil AND/OR CFP-10 - Nil IU/mL	Report	Interpretation
≥ 0.5	≥ 0.35	QuantiFERON®-TB Gold Positive	*M. tuberculosis* infection likely
< 0.5	≥ 0.35	QuantiFERON®-TB Gold Positive	*M. tuberculosis* infection likely
≥ 0.5	< 0.35	QuantiFERON®-TB Gold Negative	*M. tuberculosis* infection NOT likely
< 0.5	< 0.35	QuantiFERON®-TB Gold Indeterminate	*Result not obtained*

[1] Mitogen - Nil must be ≥ 0.5 IU/mL OR either ESAT-6 or CFP-10 minus Nil must be ≥ 0.35 IU/mL for a subject to have a valid QuantiFERON®-TB Gold result.

[2] For a patient to be considered POSITIVE for *M. tuberculosis* infection, either or both of the individual CFP-10 minus Nil and ESAT-6 minus Nil responses must be greater than or equal to 0.35 IU/mL.

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QuantiFERON - Gold vs TST

Korean med students: *n* = 99 (54 pulm TB+)

- specificity = 96% vs 49%
- sensitivity = 81% vs 78%

Italian unselected hospital pts: *n* = 318

- sensitivity = 67% vs 33%
- large number of indeterminants (*n* = 50)

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Where do we go from here?

Genotyping –

Better nucleic acid and genotypic markers needed;

Complete laboratory-based surveillance, will need legislation to coax labs into submitting isolates to a central location.

Use for program evaluation – (gold standard)?

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Where do we go from here?

QuantiFERON –

- Additional labs in Texas need to begin using IFG☎ on a routine basis.
- Large well-planned, head-on-head INF☎ studies needed.
- Additional studies on feasibility, acceptability and cost-benefit of INF☎ assays needed

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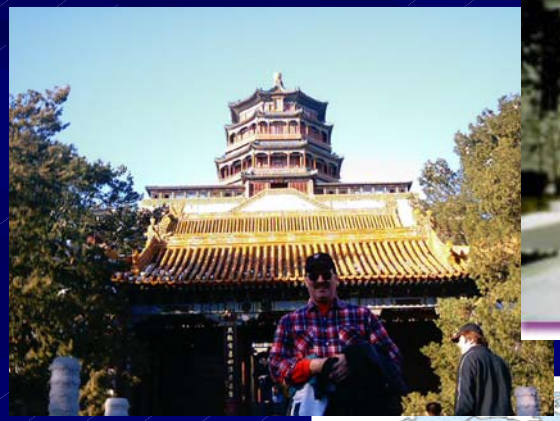
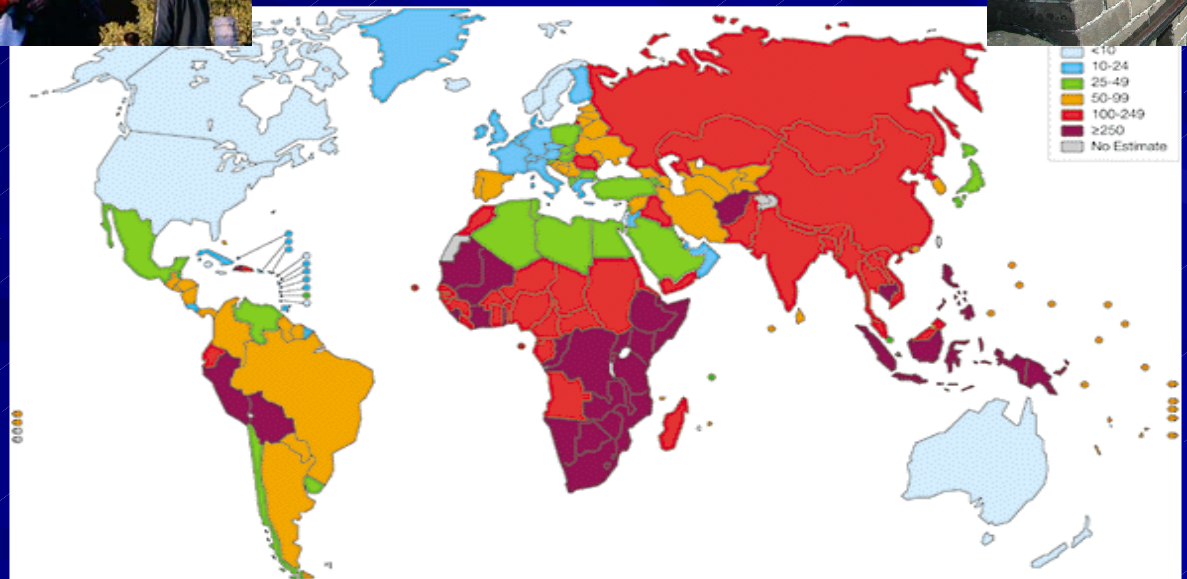
Big Thanks!





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