

# The Need for an Aspergillus Standard

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# Disclosures

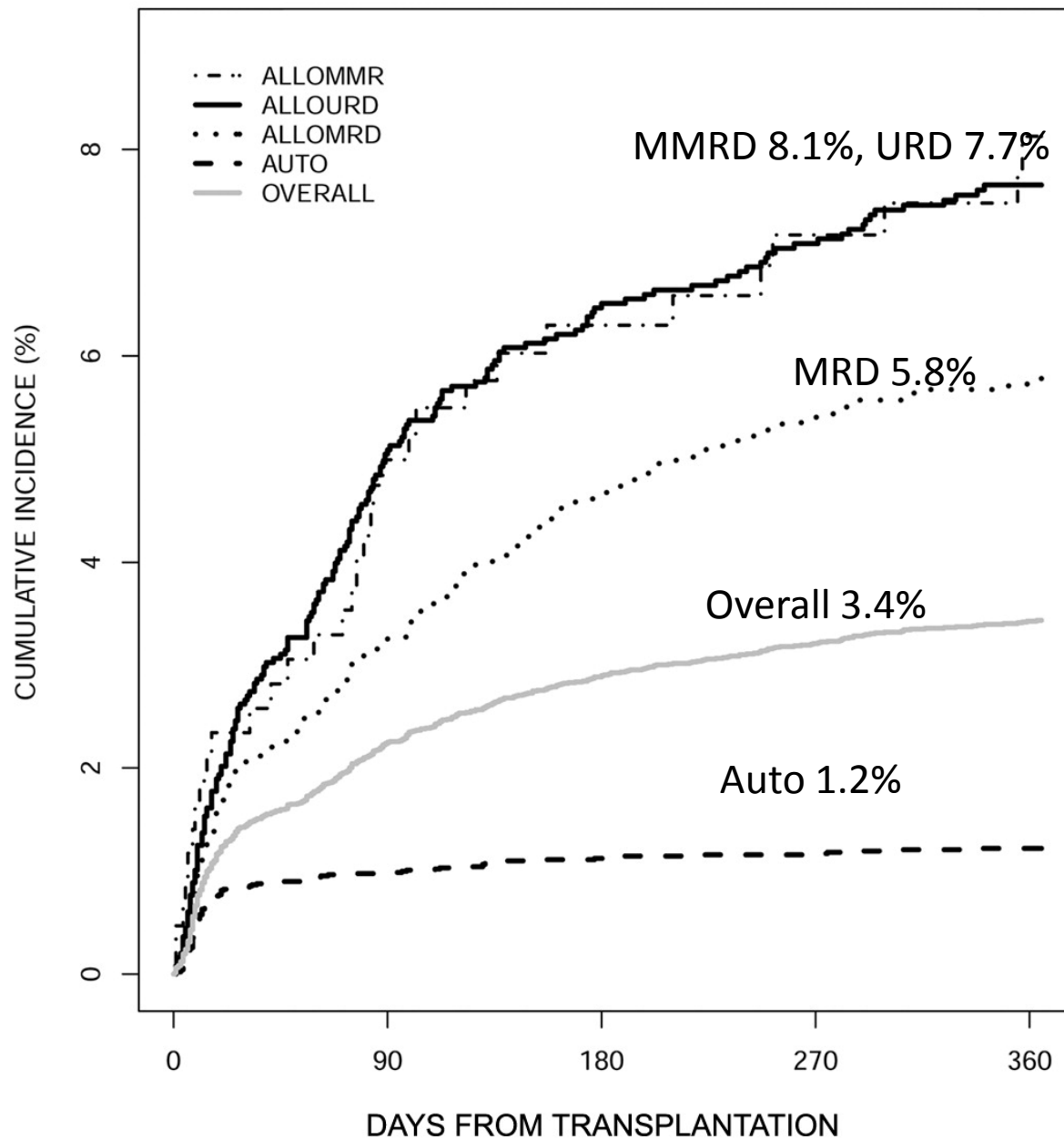
- Scientific Advisory Board: Abbott Molecular, Roche Diagnostics, GenProbe, Idaho Technologies, Quidel
- Consultant: Biotrin/DiaSorin
- Research/Clinical Trials: Qiagen, Roche

# Prospective Surveillance for Invasive Fungal Infections in Hematopoietic Stem Cell Transplant Recipients, 2001-2006: (TRANSNET Database).

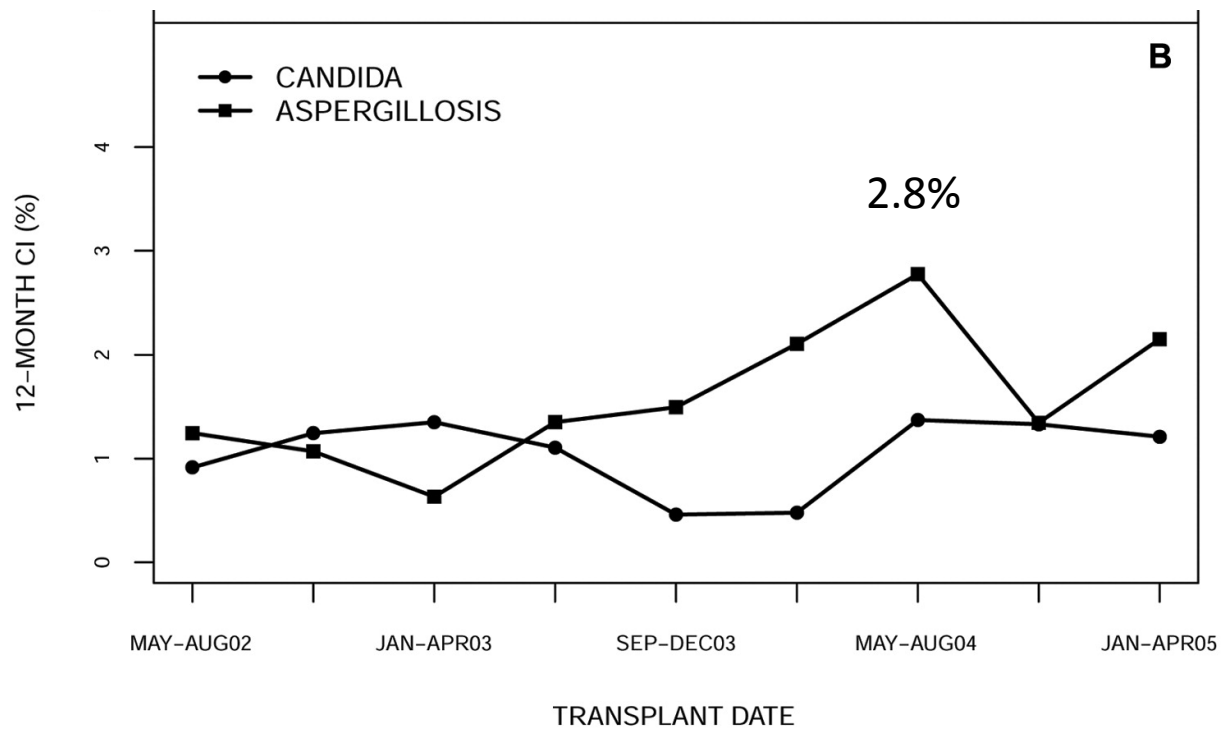
- TRANSNET sentinel surveillance system, administered by CDC and coordinating center at UAB
- Prospective surveillance 2001-2006
- 23 US academic tx centers; 22 contributed HSCTs
- Only proven and probable cases included
- Surveillance cohort: all HSCT with IFI
- Incidence cohort: HSCT underwent tx from 3/2001-9/2005

# IFI HSCT

- 983 proven and probable IFI among 875 HSCT recip
  - 21% autologous
  - 78% allogeneic
    - 38% MRD, 6% MMRD (mis-matched-related), 34% URD
- Aspergillosis 43%
  - *A. fumigatus* 44%, *A. niger* 9%, *A. flavus* 7%
- Candidiasis 28%
  - *C. albicans* 20%, *C. glabrata* 33%, *C. parapsilosis* 14%
- Zygomycosis 8%



12-month Cumulative Incidence any IFI 3.4%



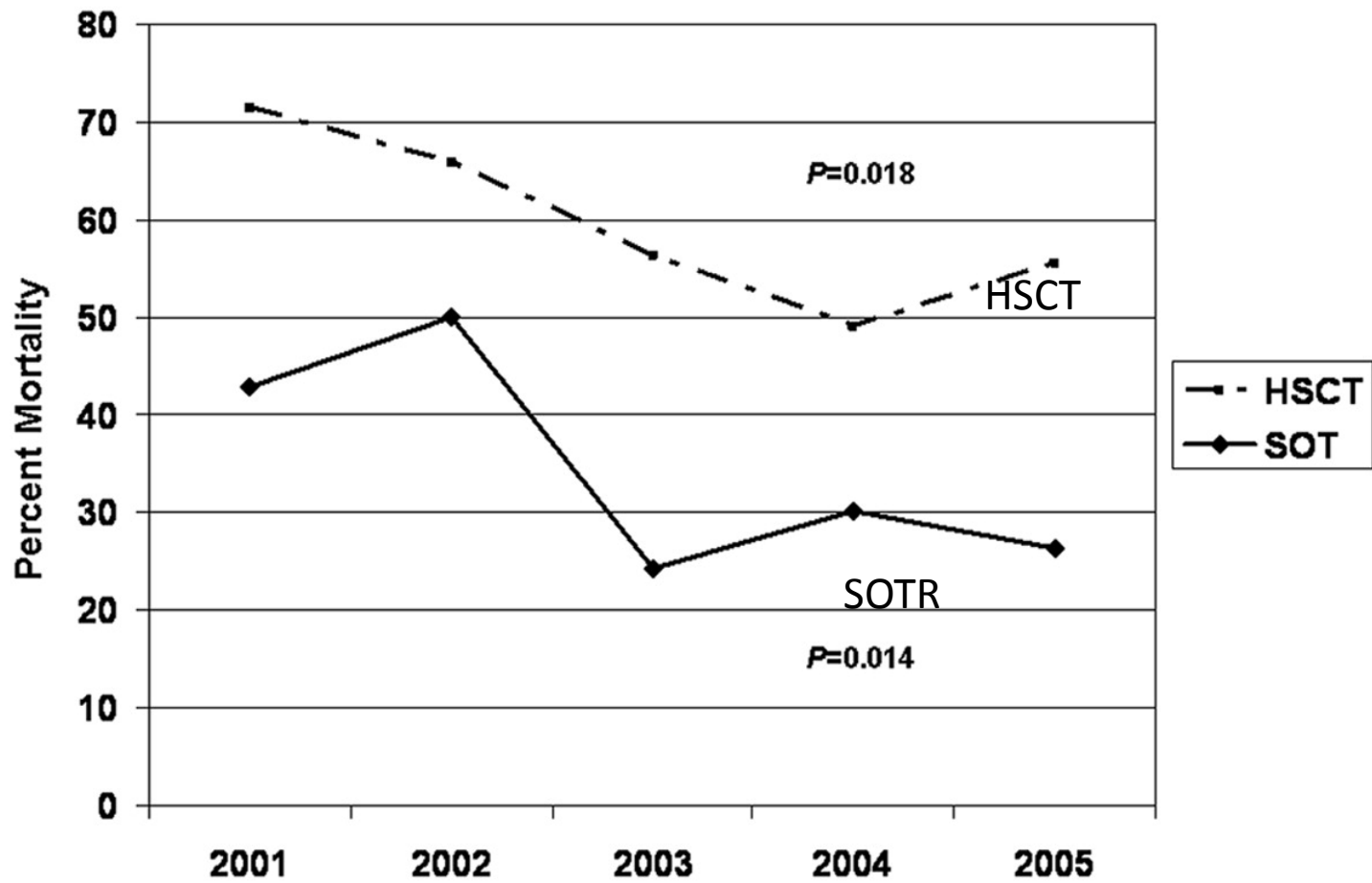
Kontoyiannis DP et al. CID 2010;50:1091-1100

**Table 2. No. (%) of Invasive Fungal Infection (IFI) Cases in the Surveillance Cohort, by Transplant Type**

IFI type	Kidney (n = 332)	Liver (n = 378)	Pancreas (n = 128)	Lung (n = 248)	Heart (n = 99)	Small bowel (n = 22)
Candidiasis	164 (49)	255 (68)	97 (76)	56 (23)	48 (49)	19 (85)
Aspergillosis	47 (14)	42 (11)	6 (5)	109 (44)	23 (23)	0 (0)
Zygomycosis	8 (2)	9 (2)	0 (0)	8 (3)	3 (3)	0 (0)
Other mold	10 (3.0)	9 (2.4)	4 (3.1)	49 (19.8)	7 (7.1)	0 (0.0)
Unspecified mold	7 (2.1)	8 (2.1)	0 (0.0)	7 (2.8)	2 (2.0)	0 (0.0)
Cryptococcosis	49 (15)	24 (6)	6 (5)	6 (2)	10 (10)	1 (5)
Endemic mycoses	33 (10)	17 (5)	8 (6)	3 (1)	3 (3)	0 (0)
Pneumocystosis	5 (1)	0 (0)	1 (1)	4 (2)	3 (3)	0 (0)
Other yeast	6 (1.8)	9 (2.4)	5 (3.9)	0 (0.0)	0 (0.0)	1 (5)
Unspecified yeast	3 (0.9)	5 (1.3)	1 (0.8)	6 (2.4)	0 (0.0)	1 (5)

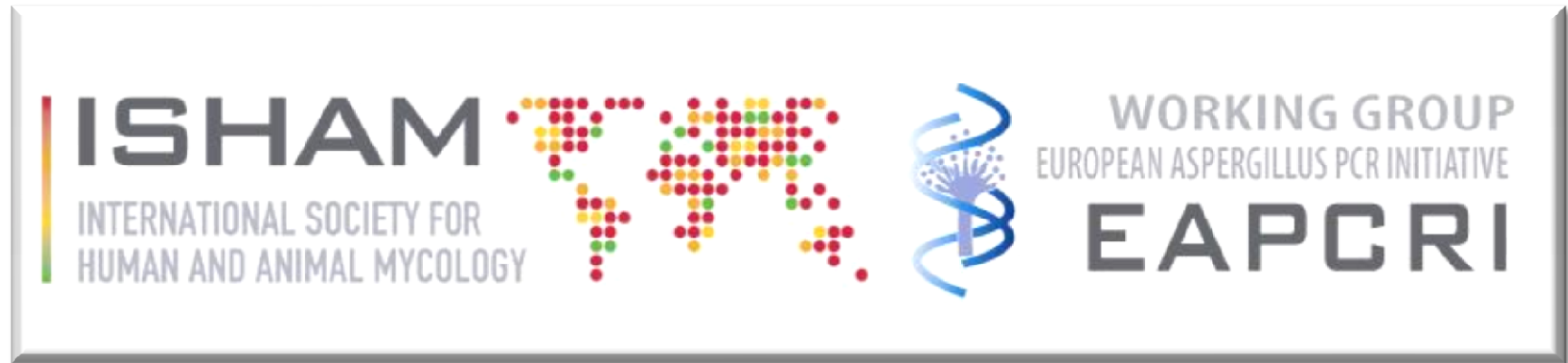
# TRANSNET DATA: Invasive Aspergillosis

## Mortality per Study Year



# Establishing the Clinical Utility of PCR

- Interest in using PCR assays for diagnosis of IA
  - Lack of standardization
  - Difficult to establish clinical utility
- What form of the organism is present clinically
  - Blood?
  - Respiratory, tissue



## European PCR Standardization Initiative: Current Status

24 laboratories participated

Dr P. Lewis White  
Public Health Wales, Microbiology Cardiff  
on behalf of the EAPCRI



## Results from 1st distribution

- DNA panel
  - 90% of centres achieved required threshold (equivalent to 50 *A. fumigatus* genomes in 100ul sample, mean Ct 35.3)
  - 45% of centres were able to detect DNA concentrations equivalent to 5 *A. fumigatus* genomes
- Whole blood panel (3ml blood spiked with conidia, <100 conidia/specimen)
  - Only 45% of centres achieving PCR threshold maintained this cut-off when extracted DNA from WB
- Centres maintaining threshold used entire specimen and bead-beating
- Nucleic acid extraction method critical

## 2nd Distribution – with recommendations

Protocol	Sensitivity	95% CI	Specificity	95% CI	DOR	95% CI
All	80.6%	68.2 – 88.9	86.3%	76.1 – 92.6	39.8	12.4 – 127.3
Compliant	88.7%	79.8 – 94.0	91.6%	79.1 – 96.9	119.9	44.9 – 319.9
Non-compliant	57.6%	37.9 – 75.2	77.2%	61.2 – 87.9	8.9	1.7 – 45.5

$\Delta$ Sensitivity: 31.1 p= 0.008,  $\Delta$ DOR: 111 p= 0.006

# Meta-Regression Analysis

- Bivariate analysis:
  - Positive correlation between sensitivity and
    - Compliant protocols
    - Bead beating
    - IC in the PCR
- Multivariate analysis
  - Positive correlation between sensitivity and
    - WBC lysis buffer
    - Bead beating
    - IC in the PCR
  - Negative correlation between sensitivity and elution volume >100ul

Frozen or fresh EDTA whole blood is acceptable

3-4mL EDTA whole blood

Threshold : 12/18

Step time

Overall Time

# The Method

Suitable commercial alternative available from Promega

Hypotonic Red Cell Lysis<sup>a</sup> (10mL):  
Incubate ambient temperature

Threshold: 13/19

15mins<sup>b</sup> x2

1<sup>st</sup> Wash decant supernatant

5mins

Centrifugation 3000xg

10mins

2<sup>nd</sup> Wash decant supernatant

5mins

1h

Recombinant Proteinase K preferred

White Cell Lysis<sup>a</sup> (1mL) Incubate 65°C

Threshold: 9/12

45mins

Centrifugation 5000 xg

10mins

1h55

Decant 0.8ml supernatant. Add glass beads

10mins

Ceramic or acid washed glass beads (710-1180µm)

Bead-beating

Threshold: 14/18

1min

2h06

Pulse centrifuge

Transfer 0.2mL supernatant or wash with 0.2ml molecular grade water

5mins

DNA purification/ppt can be performed using automated or manual processing using the commercial kits highlighted in DNA strategies 1, 2, 6-11, 15 and 16. All reagents should be batch screened for fungal contamination.

DNA purification/ppt

Up to 1h

Elution Volume <100µL

Threshold: 14/18

3h15

<sup>a</sup>Lysis buffer as described previously (6)

<sup>b</sup>Involves 5 minutes processing time and 10 minutes incubation time. When processing frozen specimens it is not necessary to incubate.

## **The current EAPCRI recommendations are:**

**All recommendations apply to EDTA whole blood.**

- 1. A minimum of 3 ml of blood needs to be extracted**
- 2. Red and white cell lysis is required**
- 3. Bead-beating for lysis of fungal cells**
- 4. A real time PCR platform using a multi-copy target and species / genus-specific hybridization probes**
- 5. Analysis of all specimens in duplicate, if discrepancy occurs, repeat on identical DNA extract**
- 6. An Internal control PCR is essential**
  - 1. Preferably non-human target**
  - 2. Concentrations equivalent to typical fungal burdens**
- 7. The use of controls for DNA extraction and PCR assay is essential**
- 8. Elution volume <100µl**
- 9. EDTA is the only anticoagulant to be used, sodium citrate and heparin should not be used**
- 10. All batches of reagents should be screened for contamination prior to use**

# Aspergillus Technology Consortium (AsTeC)

- NIH funded contact facilitate diagnostics for IA
  - Wingard, PI (Univ Florida), Alexander, Co-PI (Duke), Baden (Harvard/BWH/DFCI), Caliendo and Lyon (Emory), Wheat (MiraVista), Denning (Univ Manchester), Clancy and Nguyen (Univ Pgh)
  - Repository of samples on cases of proven and probable IA, and those at high risk for IA
- Repository available to companies or indiv with tests
  - Contact Dr. Wingard or [www.asteccdiagnostics.org](http://www.asteccdiagnostics.org)

Same test (same run) using the IAAM (T Patterson)  
and Emory calibrators (standard curve)

Sample Number	IAAM (ng/ul)	Emory (ng/ul)	Fold Difference
1	7.7	117.3	15.2
2	1.1	17.7	16.1
3	$8 \times 10^{-2}$	1.5	18.8
4	$9 \times 10^{-3}$	$1.8 \times 10^{-1}$	20
5	$9.1 \times 10^{-4}$	$1.9 \times 10^{-2}$	20.1
6	$1.2 \times 10^{-4}$	$3.0 \times 10^{-3}$	18.8
7	$1.2 \times 10^{-5}$	$2.9 \times 10^{-4}$	24.2

# Aspergillus Calibrator

- Calibrator material
  - Purified nucleic acid, rather than biological material
  - Ultimately a biological standard is needed
- Will not evaluate the extraction methods
  - Based on White et al, this is critical

# AsTeC DNA Calibrator: Limiting Dilution

Purified from cultured *A. fumigatus* (AF293)

Each laboratory tested 6 concentrations; 6 or 10 replicates  
63% hit rate = 1 copy/rxn (probit), calculate concentration of stock,  
corrected for 35 copies per genome (18s RNA gene)

Emory Calibrator	Units/ml
Laboratory 1	$5.11 \times 10^8$
Laboratory 2	$8.00 \times 10^8$
Laboratory 3	$0.97 \times 10^8$
Laboratory 4	$0.74 \times 10^8$
Mean	$3.71 \times 10^8$

Range in values ~10 fold

# Calibrator Stability (AsTeC Calibrator)

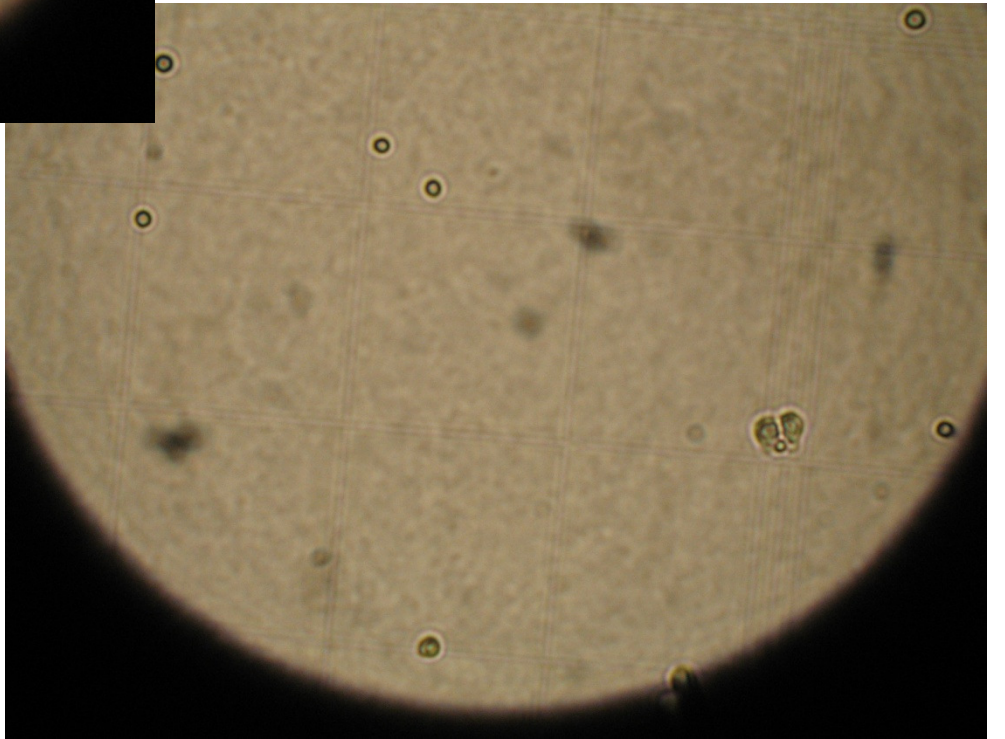
Freeze/Thaw Cycles						
Replicate	0	1	2	3	5	10
1	35.0	33.7	34.1	33.9	35.3	34.0
2	34.0	33.8	34.6	34.3	35.0	34.7
3	33.5	36.0	33.8	34.3	33.8	33.6
4	34.6	33.8	35.7	34.0	34.4	34.6
5	33.6	35.4	34.8	34.6	35.1	34.6
6	34.5	34.5	33.7	33.7	35.0	34.7
7	36.2	34.5	35.2	33.5	34.4	34.2
8	34.3	34.3	35.8	33.9	34.6	34.8
9	33.3	33.2	34.2	33.5	34.7	35.7
10	34.6	34.1	34.5	34.1	34.8	33.8
<b>AVE Ct</b>	<b>34.3</b>	<b>34.3</b>	<b>34.7</b>	<b>34.0</b>	<b>34.7</b>	<b>34.5</b>
<b>STD DEV</b>	<b>0.80</b>	<b>0.79</b>	<b>0.70</b>	<b>0.35</b>	<b>0.42</b>	<b>0.57</b>

## Storage Stability (AsTeC Calibrator)

Replicate	0	1 MOS	3 MOS	6 MOS	1 year	2 Year
1	35.0	35.2	35.4	35.3		
2	34.0	36.7	34.8	35.4		
3	33.5	35.4	36.0	34.8		
4	34.6	34.9	34.7	35.8		
5	33.6	34.3	35.2	35.1		
6	34.5	36.0	35.2	33.9		
7	36.2	35.7	37.5	34.4		
8	34.3	35.8	34.8	36.0		
9	33.3	35.2	35.3	34.7		
10	34.6	35.7	35.3	34.9		
AVE (SD)	34.3 (0.8)	35.5 (0.6)	35.4 (0.8)	35.0 (0.6)		

# Next Steps

- Expanding number of sites evaluating calibrator
  - 12 sites in the US and Europe (EAPCRI)
  - Assign a copy number
- Material will be available through AsTeC to anyone wishing to test the material
- “Biological” calibrator
  - Includes the extraction method
  - Germlings or conidia in a matrix
  - Reflect actual clinical specimen: germlings
  - Ease of production and stability: swollen conidia



# Acknowledgements I

## The EAPCRI Steering Group

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## The EAPCRI Laboratory Working Group

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