A proposed standard for Aspergillus PCR

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IA has major medical and socio-economic impact in certain patient cohorts

- Annual cost of treating fungal infections in Europe > 100 hundred million Euro annually.
- In-hospital stays for patients with IA was found to be a median of 17.7 days longer than uninfected patients
- Costs were estimated to be 75000 Euros higher per patient
- In the USA, IA occurs in approximately in 10000 patients annually

- Diagnosis of IA shows poor sensitivities, depending on patient cohort
- No „golden standard“ of diagnostic assay available
- Antigen detection (e. g. galactomannan) shows cross-reactivity, CT scans might be unspecific, blood cultures not of diagnostic value
- No commercially available well evaluated molecular assays

→ There is an **obvious need** for standardization of Aspergillus molecular-based detection from whole blood samples:

- DNA extraction procedure
- PCR assays
- Clinical studies

In June 2006, during the 16th ISHAM meeting in Paris, the **EAPCRI was born**

→ Laboratory Working Party: initial meeting in September 2006

The Members of the core group of the EAPCRI LWP:

- Juergen Loeffler (Chair), Wuerzburg
- Stephane Bretagne, Paris
- Niklas Finnström, Cepheid, Toulouse
- Willem Melchers, Nijmegen
- Lewis White, Cardiff
- Lena Klingspor, Stockholm
- Elaine Mc Culloch, Glasgow
- Bettina Schulz, Berlin

Two different panels were distributed to the

<u>8 laboratories</u> of the core group:

- The whole blood panel:
  - 10 specimens
  - 4ml EDTA Horse Blood
  - Eight positive and two negative
  - Range $>10^4$ conidia to 10 conidia in total

- The DNA panel:
  - 10 specimens
  - Serially diluted *A. fumigatus* DNA
  - Eight positive and two molecular grade waters
  - Expected 100% cut off = -5 dilution

Summary of Whole Blood results

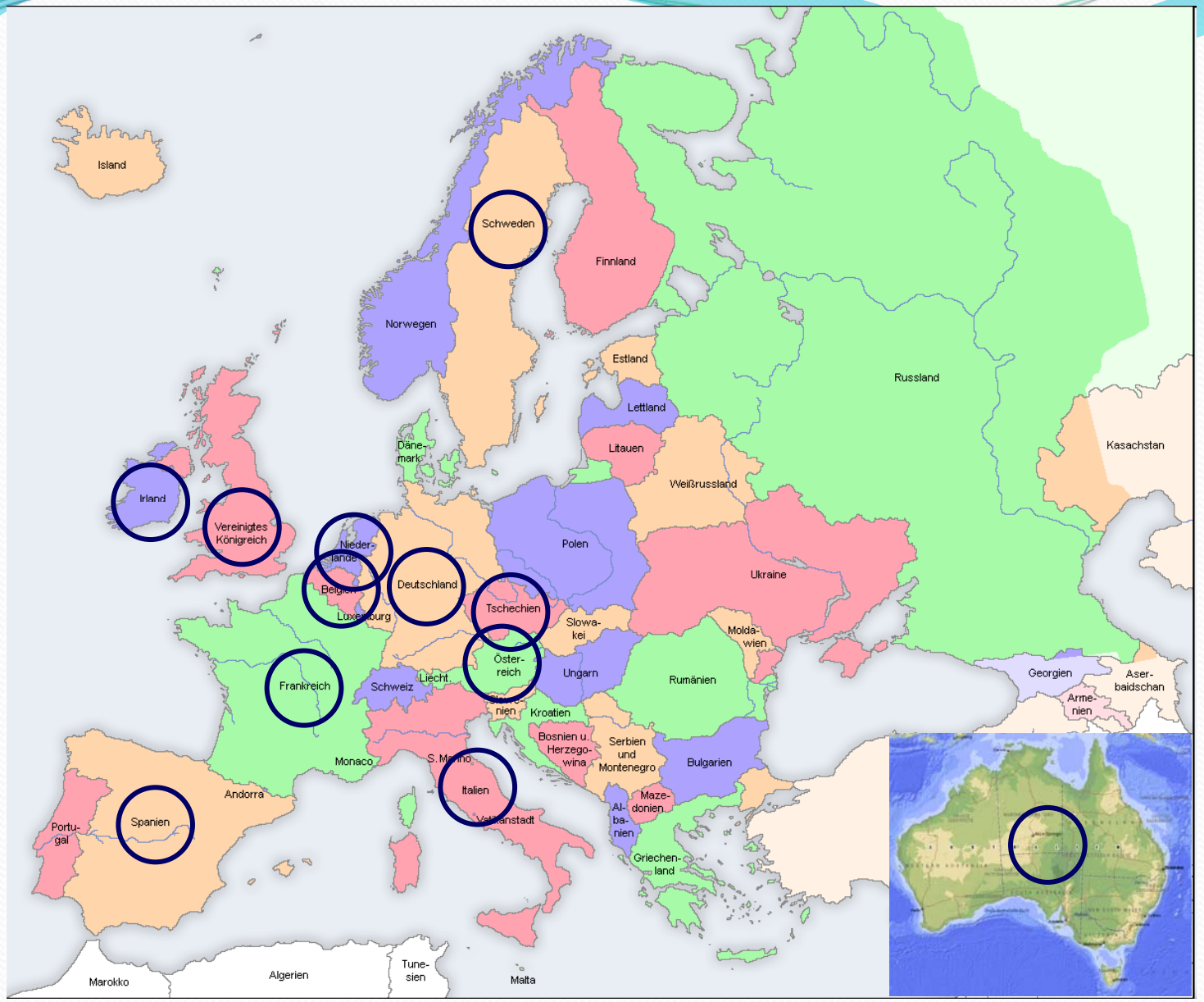
- Overall mean sensitivity: 60.9 – 70.3% (Range 37.5 – 100%)
  - 7 centres up to 50%
  - 5 centres up to 70%
  - 4 centres up to 80%
  - 3 centres up to 100%
- Overall mean specificity: 78.6% (Range 0 – 100%)
- 100% of assays detected 500 conidia (250 conidia/ml)
- 68% of assays detected <100 conidia

Expanded Distribution

- 15 additional centres throughout Europe and 1 centre in Australia
- Centres have claimed to have fungal PCR experiences
- Same format as last panel

ISHAM
INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY

WORKING GROUP
EUROPEAN ASPERGILLUS PCR INITIATIVE
EAPCRI



Results of DNA Panel – PCR performance

- Overall mean sensitivity: 66.9 – 69.4% (Range 0 – 100%)
  - 19 centres >60%
  - 10 centres >70%
  - 5 centres >80%, 2 with multiple positives
- Overall mean specificity: 95 – 97.5% (Range 50 – 100%)
- 95% of assays detected down to predicted cut-off

Results of Whole Blood results – Extraction performance

- Overall mean sensitivity: 47.2 – 57.2% (Range 0 – 100%)
  - 15 centres up to 50%
  - 8 centres up to 70%
  - 6 centres up to 80%
  - 3 centres up to 100% (none with multiple positives)
- Overall mean specificity: 90% (Range 0 – 100%)
- 70% of assays detected 500 conidia

The 4th panel – A recommended protocol to be used by all centres

May 2008:

- All 3 ml blood to be used
- Bead-beating release of fungal DNA
- real-time PCR assay
- internal control
- 3 replicates

Specimen	Total conidia	Conidia/ml	Expected result
1	1000	333	Positive
2	0	0	Negative
3	10	3.3	Unknown
4	25	8	Unknown
5	50	16-17	Unknown
6	100	33.3	Positive
7	0	0	Negative
8	75	25	unknown
9	500	167	Positive
10	0	0	Negative

Summary of results

- The overall sensitivity: 76.6%
- Range of sensitivities: 28.6% - 100%
- 73.7% centres detected 100 CFU (33 CFU/ml)

- The overall specificity: 89%
- Range of specificity: 11.1% - 100%

Statistical analysis:

⟶ Defintion of distinct groups of laboratories

- laboratories, which followed protocol strictly (n = 12)
- laboratories, which followed protocol partially (n = 3)
- laboratories, which did not follow an acceptable protocol (n = 6)

„The professionals“

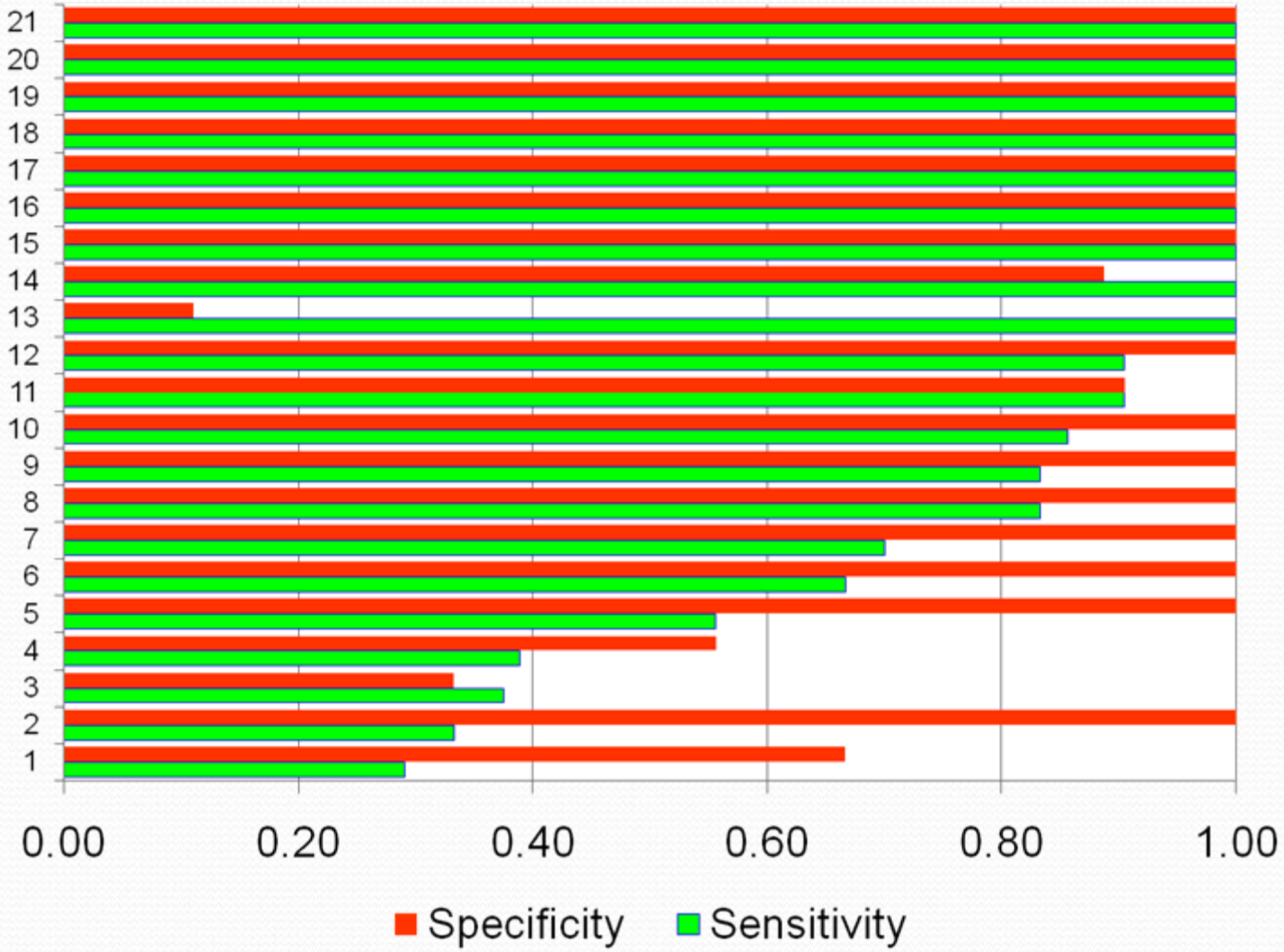
<u>Statistical analyses:</u> Carlo Mengoli, University of Padua and Mario Cruciani, University of Verona:

- **Sensitivities**
- **Specificities**
- **Diagnostic Odds Ratios**
- **Inverse Variance Weighing Method**
- **Linear Regression Analysis**
- **Restricted Maximum Likelihood Regression**

12 different PCR methodologies

	Purification of fungal DNA	Gene target	PCR format
1	High Pure Kit (Roche)	18S gene	FAM TAMRA probe
2	EZ1 (Qiagen)	18S gene	FAM TAMRA probes
3	GeneXpert (Cepheid)	18S gene	FAM TAMRA probes
4	High Pure Kit (Roche)	18S gene	FAM TAMRA probes
5	MagnaPure DNA Kit I	18S gene	FRET probes
6	Qiagen DNeasy Tissue	18S gene	FRET probes
7	Phenol-chloroform	18S gene	nested
8	QIAmp Blood Kit (Qiagen)	28S gene	FAM TAMRA probes
9	SeptiFast	ITS gene	FRET probes
10	QIAmp DNA Mini Kit (Qiagen)	ITS1 gene	FAM Molecular Beacons
11	SeptiFast Kit (Roche)	ITS2 gene	LC640, LC705, melting curves
12	ZymoSpin Filter tubes	ITS2 gene	TaqMan probes

4th panel – performance of participants

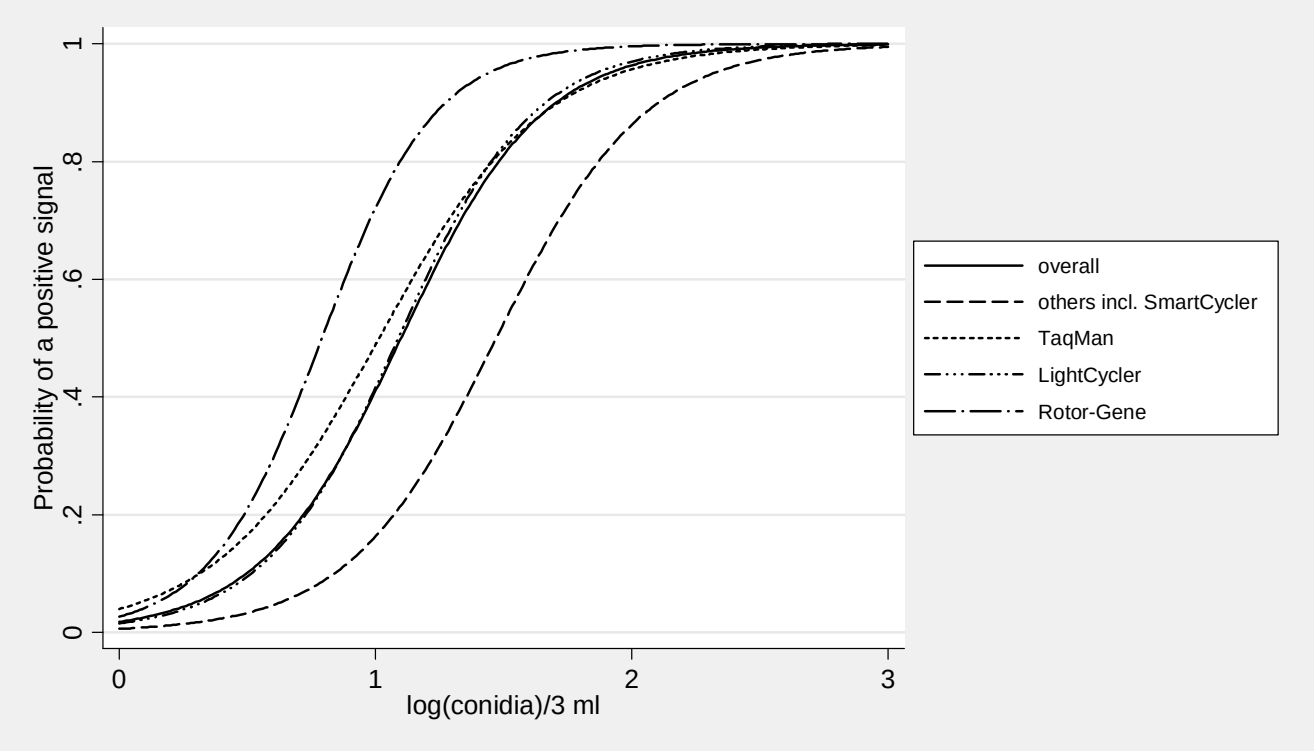


4th panel – performance of participants

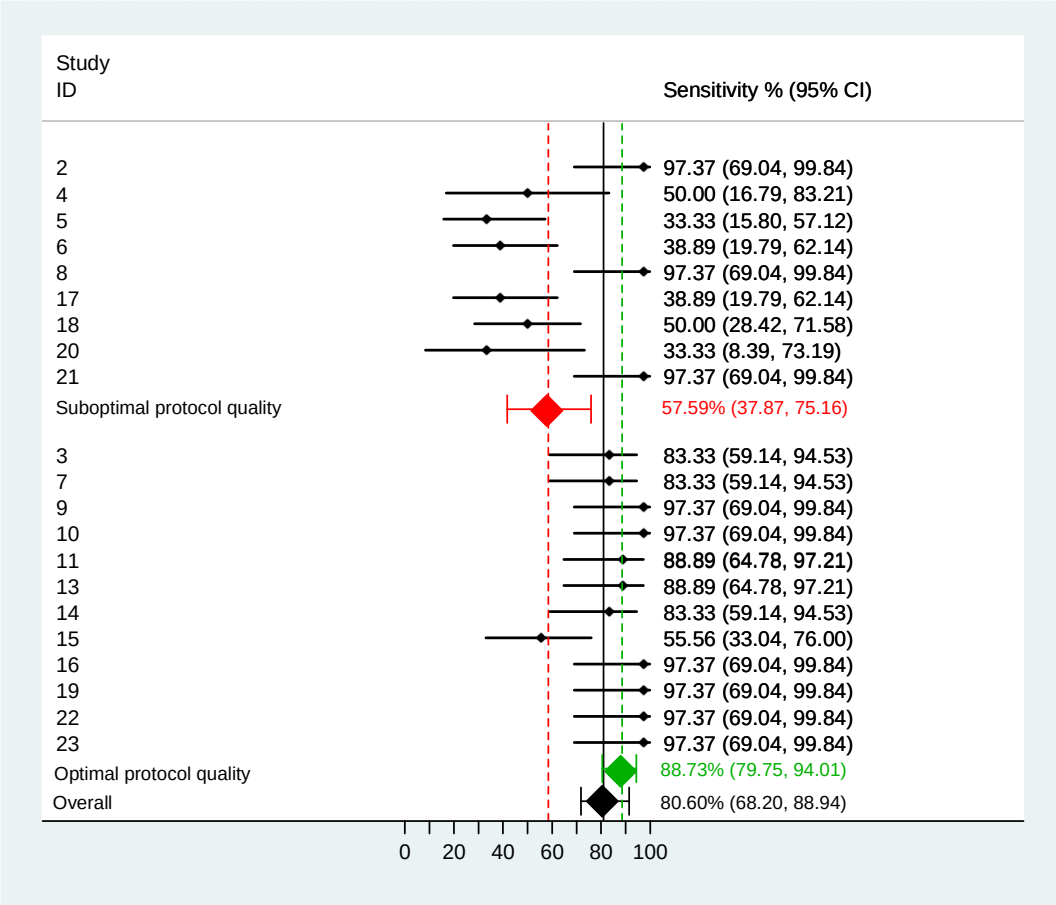
Mean performance statistics for the 2nd EAPCRI Whole Blood Panel.

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
Optimal	88.7%	79.8 – 94.0	91.6%	79.1 – 96.9	119.9	44.9 – 319.9
Sub-optimal	57.6%	37.9 – 75.2	77.2%	61.2 – 87.9	8.9	1.7 – 45.5

The probability of a positive *Aspergillus* PCR signal from EDTA whole blood spiked with varying fungal burden



Forest plot of sensitivity for each centre (red=suboptimal, green=optimal, black=overall)



Bivariate meta-regression analysis between logit sensitivity and the additional covariates

	All centres		Centres with 100% specificity	
logit sensitivity	t	P	t	P
Optimal protocol	2.98	0.008	2.69	0.018
Blood volume used	-	NS	-	NS
RCLB	-	NS	-	NS
WCLB	-	NS	-	NS
NaOH	-	NS	-	NS
Beads	2.65	0.016	2.59	0.023
Purification	-	NS	-	NS
Manual Steps >9	-	NS	-	NS
ITS	-	NS	-	NS
18S	-3.56	0.002	-	NS
28S	-	NS	-	NS
Internal control	2.79	0.012	2.85	0.015
Elution volume	-	NS	-	NS

Multivariate meta-regression analysis between logit sensitivity and the additional covariates

	Centres with 100% specificity	
logit sensitivity	t	P
Optimal protocol	-	N.S
Blood volume used	-	NS
RCLB	-	NS
WCLB	2.95	0.018
NaOH	-	NS
Beads	2.57	0.033
Purification	-	NS
Manual Steps >9	-	NS
ITS	-	NS
18S	-	NS
28S	-	NS
Internal control	2.25	0.054
Elution volume	-2.53	0.035

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. Bead-beating is required for lysis of fungal cells
3. A real time PCR platform using a multi-copy target and species / genus-specific hybridization probes
4. Analysis of all specimens in duplicate, if discrepancy occurs, repeat on identical DNA extract
5. An Internal control PCR is essential (preferably non-human)
6. The use of a negative control for DNA extraction and PCR assay is required
7. Elution volume <100µl
8. EDTA is the only anticoagulant to be used, sodium citrate and heparin should not be used
9. Some commercial products have been linked with fungal contamination. All batches of reagents should be screened for possible contamination prior to use

Acknowledgements I

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The EAPCRI Laboratory Working Group

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Acknowledgements II

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