Improved testing for the pathogenic fungi *Aspergillus fumigatus*

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Aims:

- What is *Aspergillus fumigatus?*
- Why is it a problem for the waste industry?
- Current environmental monitoring methods
- New and improved testing: RNA extraction
- New and improved testing: RT-qPCR assay
- What next?

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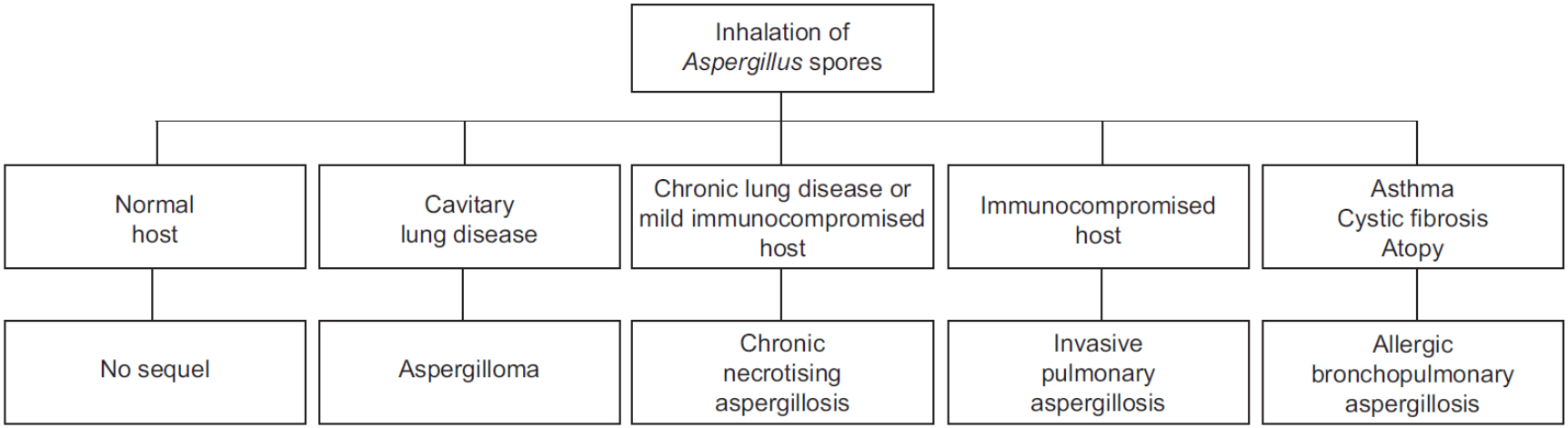
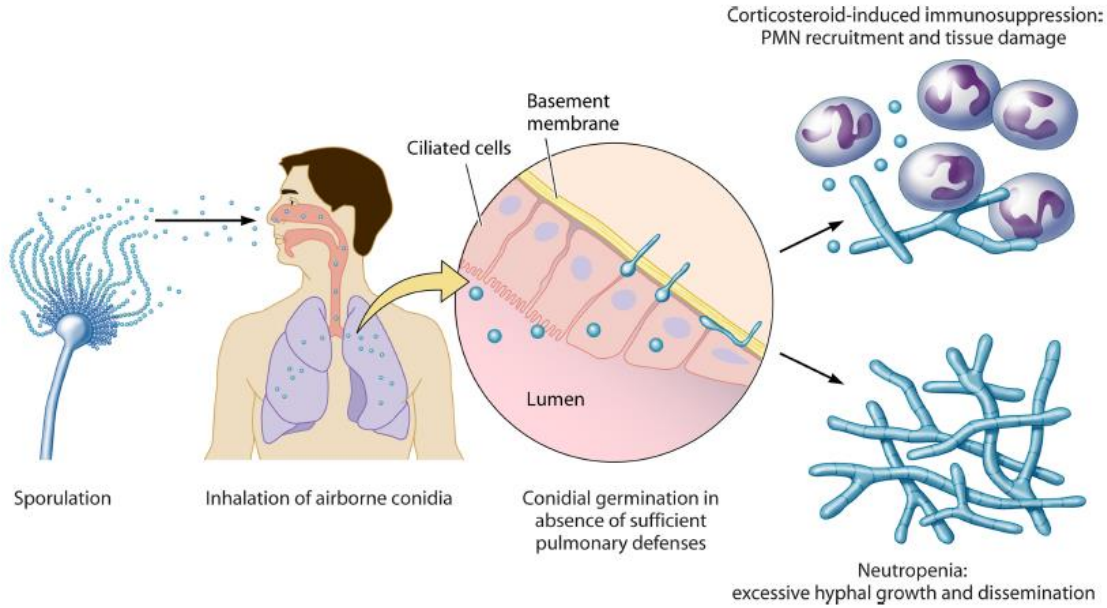
Aspergillus fumigatus: Leading cause of invasive aspergillosis





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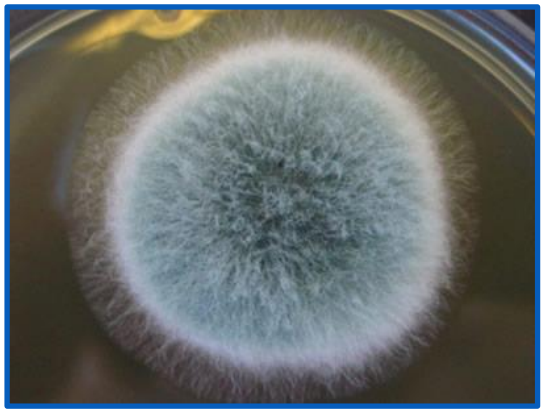
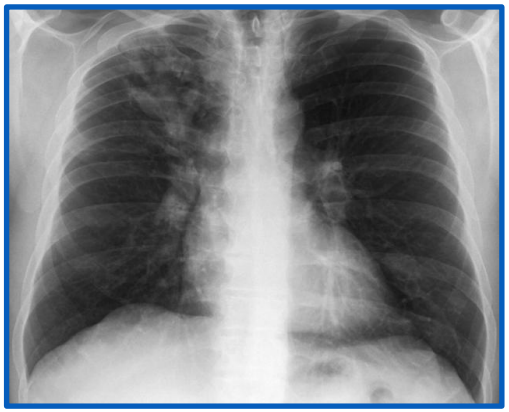
Implications for the waste industry





Case 1

- **Coughing** with expectoration of rubbery brown casts and blood from the low respir

Case 2

s

ADVISED NOT TO WORK WITH COMPOST

Existing allergic rhinitis and asthma

lying sarcoidosis

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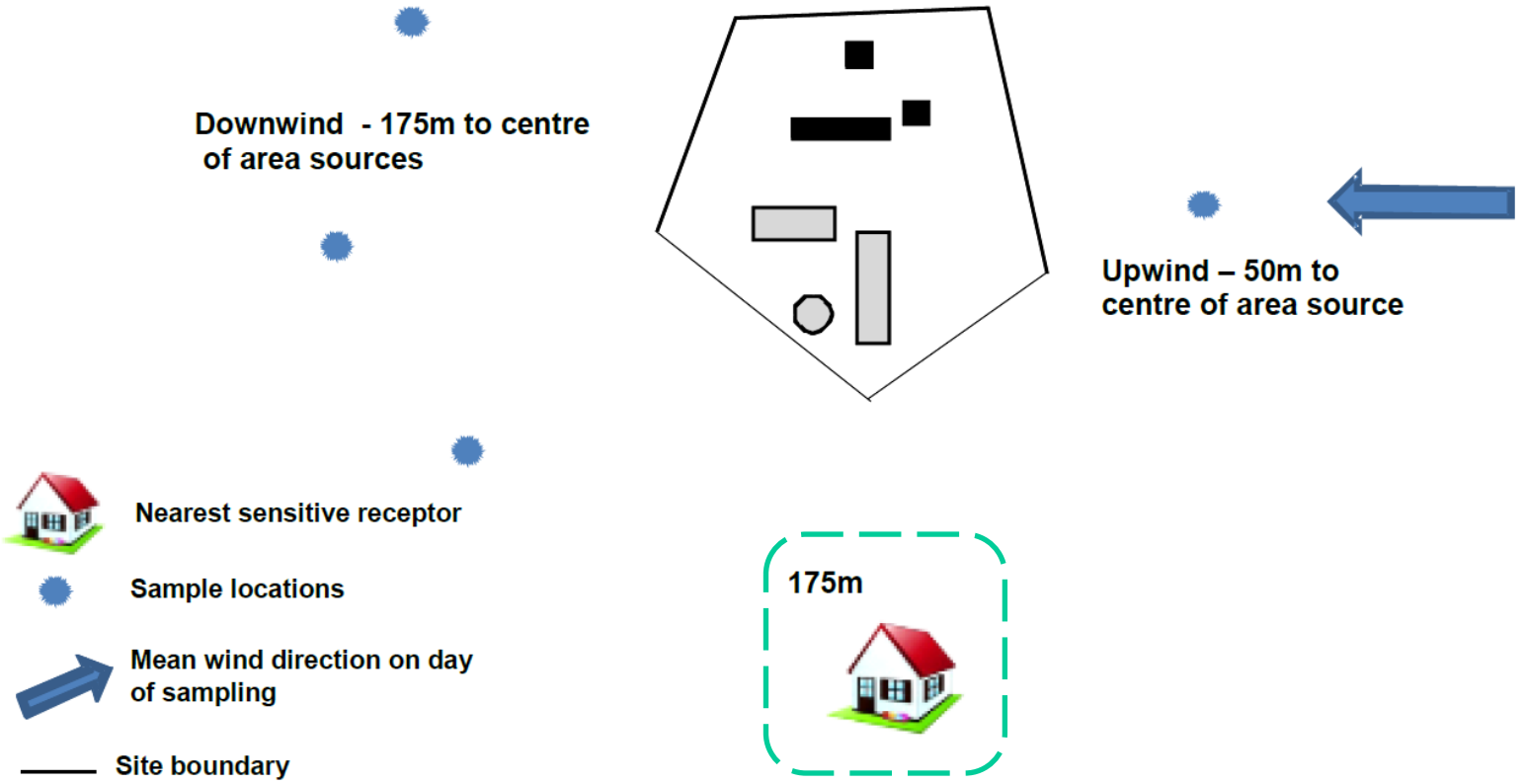
Implications for the waste industry



Sensitive receptor: nearest place to the permitted activities where people are likely to be for prolonged periods (250 m).

Current bioaerosol monitoring methods

Impaction

Filtration

Impingement

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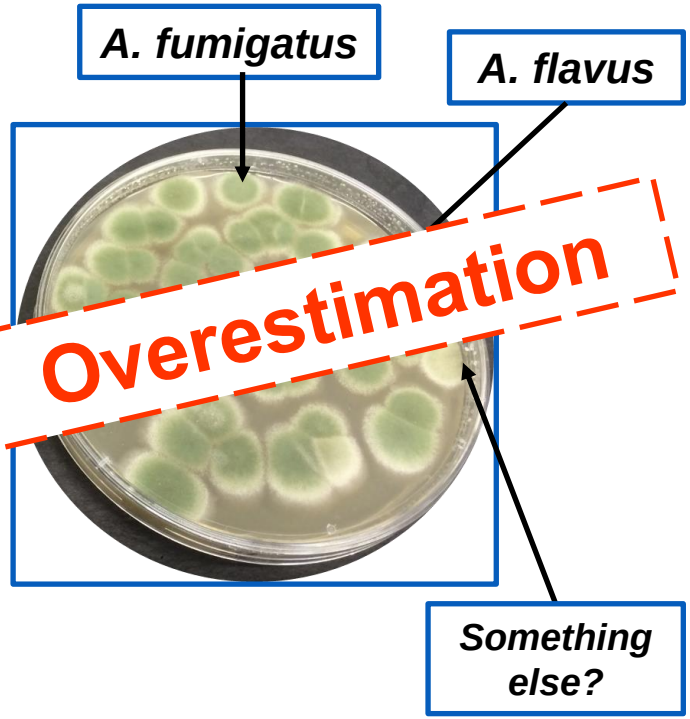
Current bioaerosol monitoring methods

- Only 10% of microorganisms are culturable
- Stressed microorganisms may not grow on SELECTIVE media
- VBNC microorganisms won't grow at all
- Current sampling techniques come with their own stressors

Underestimation



Overestimation

Why select for *A. fumigatus*?

A. fumigatus causes 90%
of all Aspergillosis cases

Environment agency stipulate AF in permits

Correct antimicrobial treatment depends on correct diagnosis

Single assay for both purposes

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New and improved testing

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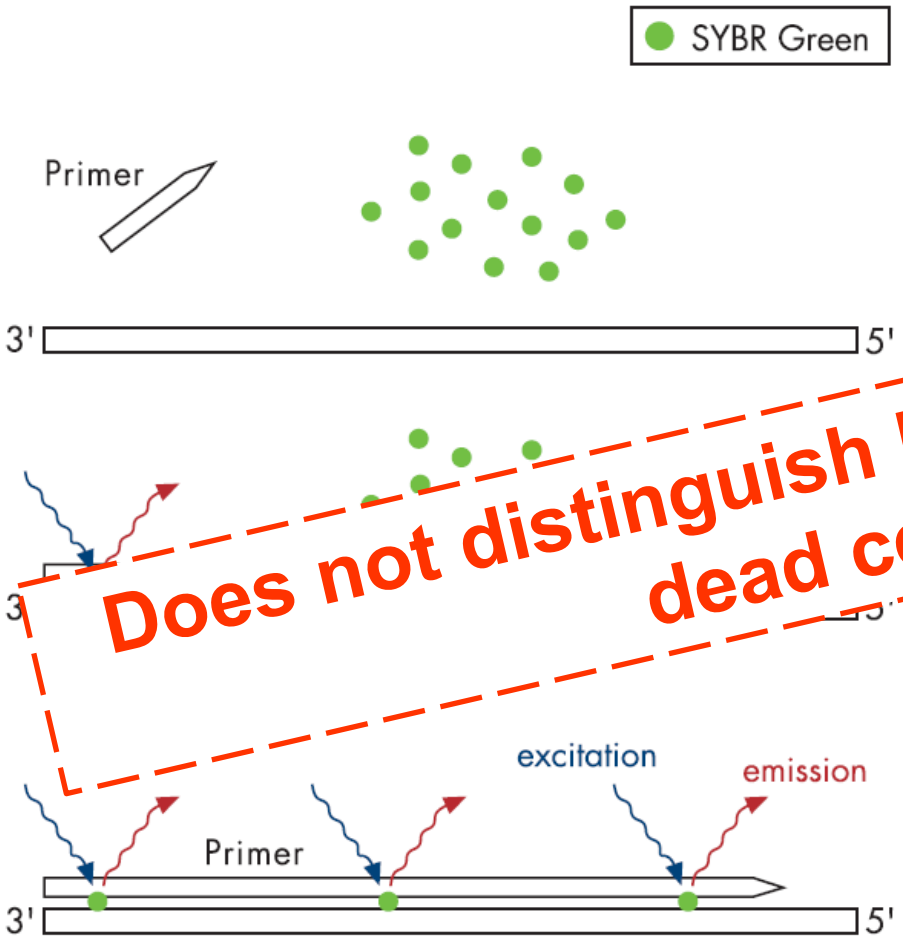
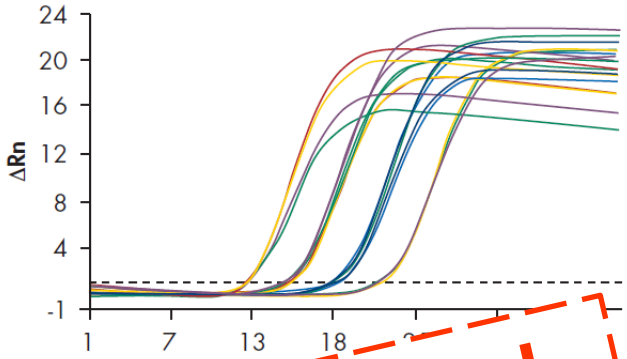
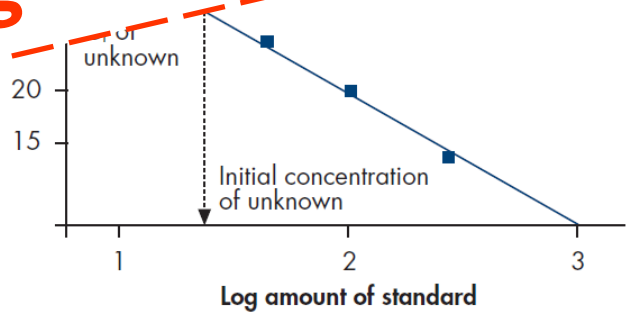
Quantitative PCR







Does not distinguish between live and dead cells

Figure 1. SYBR Green principle. Principle of SYBR Green-based detection of PCR products in real-time PCR.

Ref: Qiagen, 2010.

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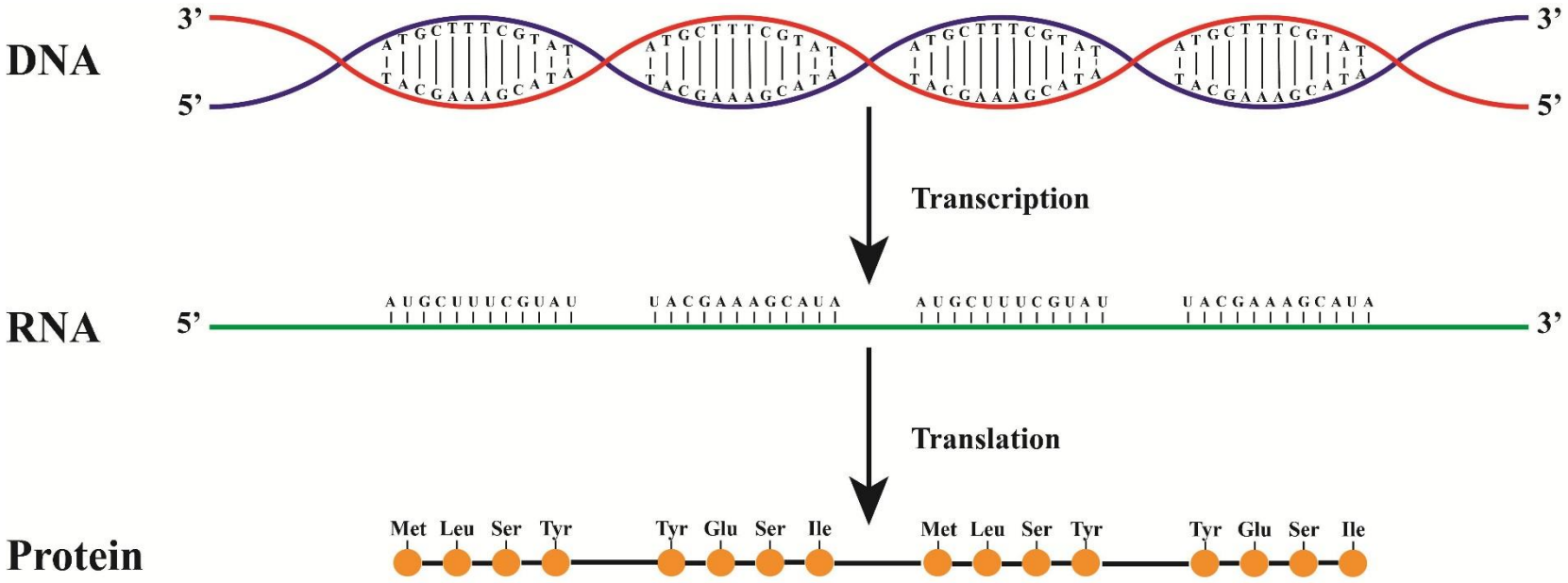
Reverse transcriptase-qPCR



RNA → Reverse transcribed → **cDNA** → **qPCR**

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New and improved testing

Two objectives:

- Extract RNA from bioaerosols
- Design a reverse transcriptase – quantitative PCR assay specific for *A. fumigatus*

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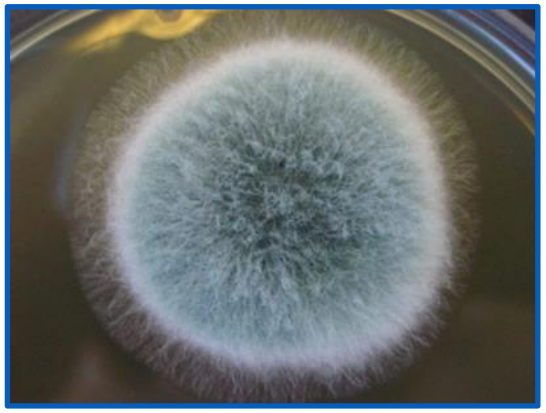
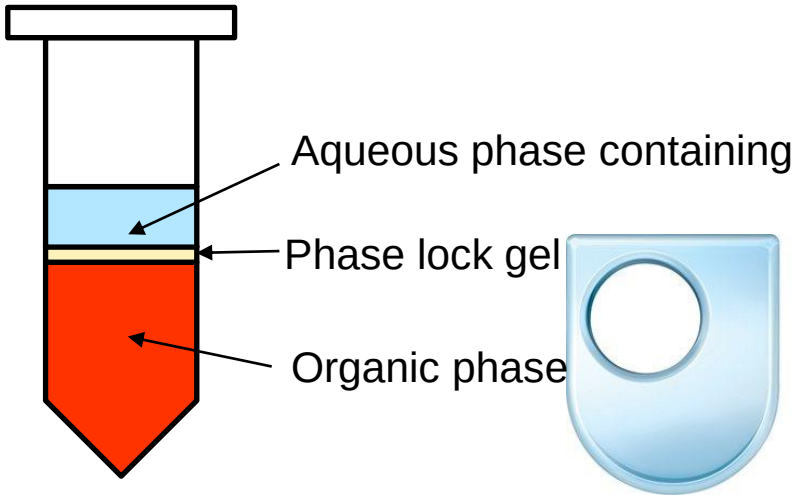
RNA extraction from pure culture

- Freeze-th… …uction
  …o Red Kit

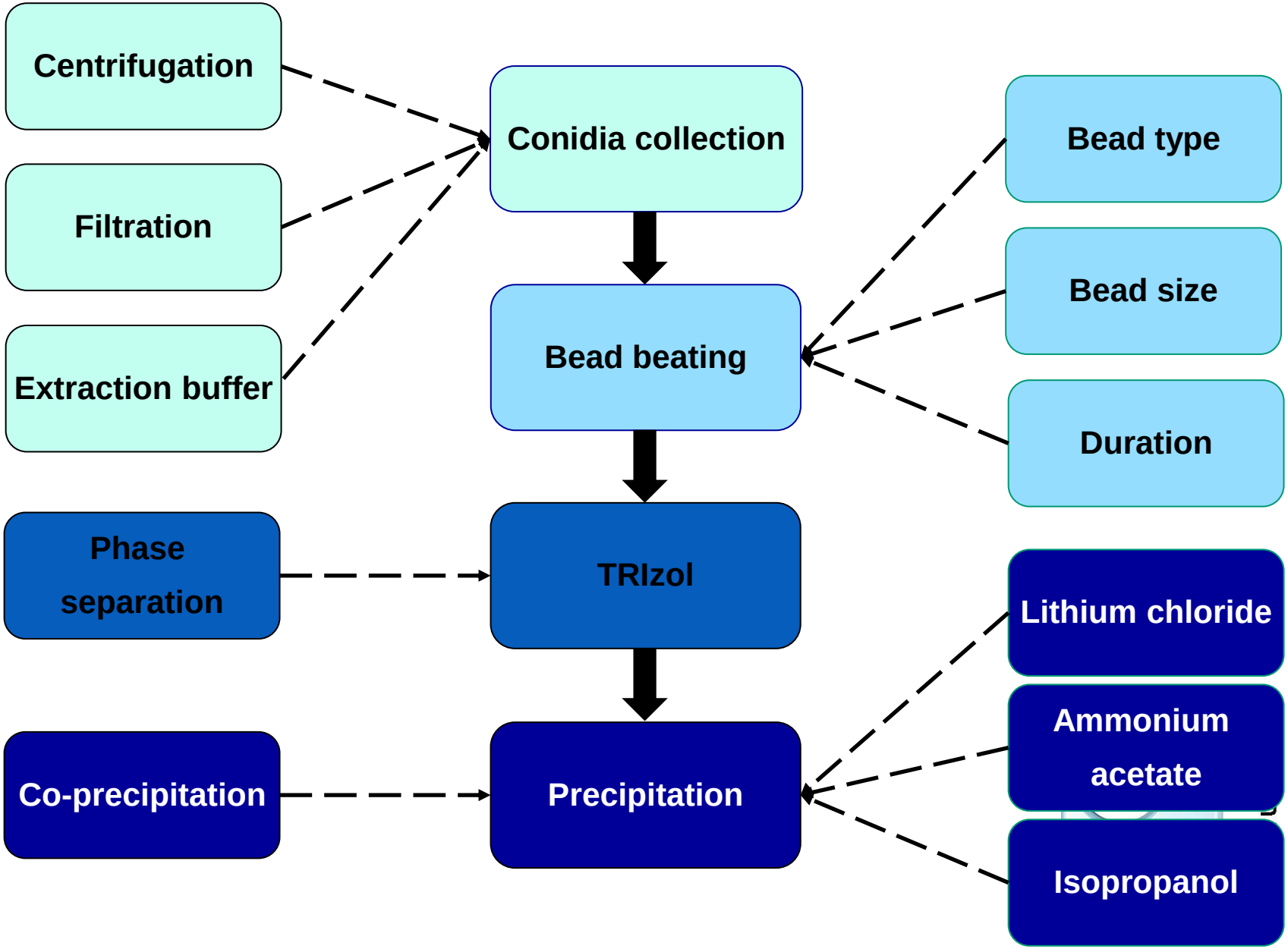
Unable to break the conidial cell wall



Phase lock tubes

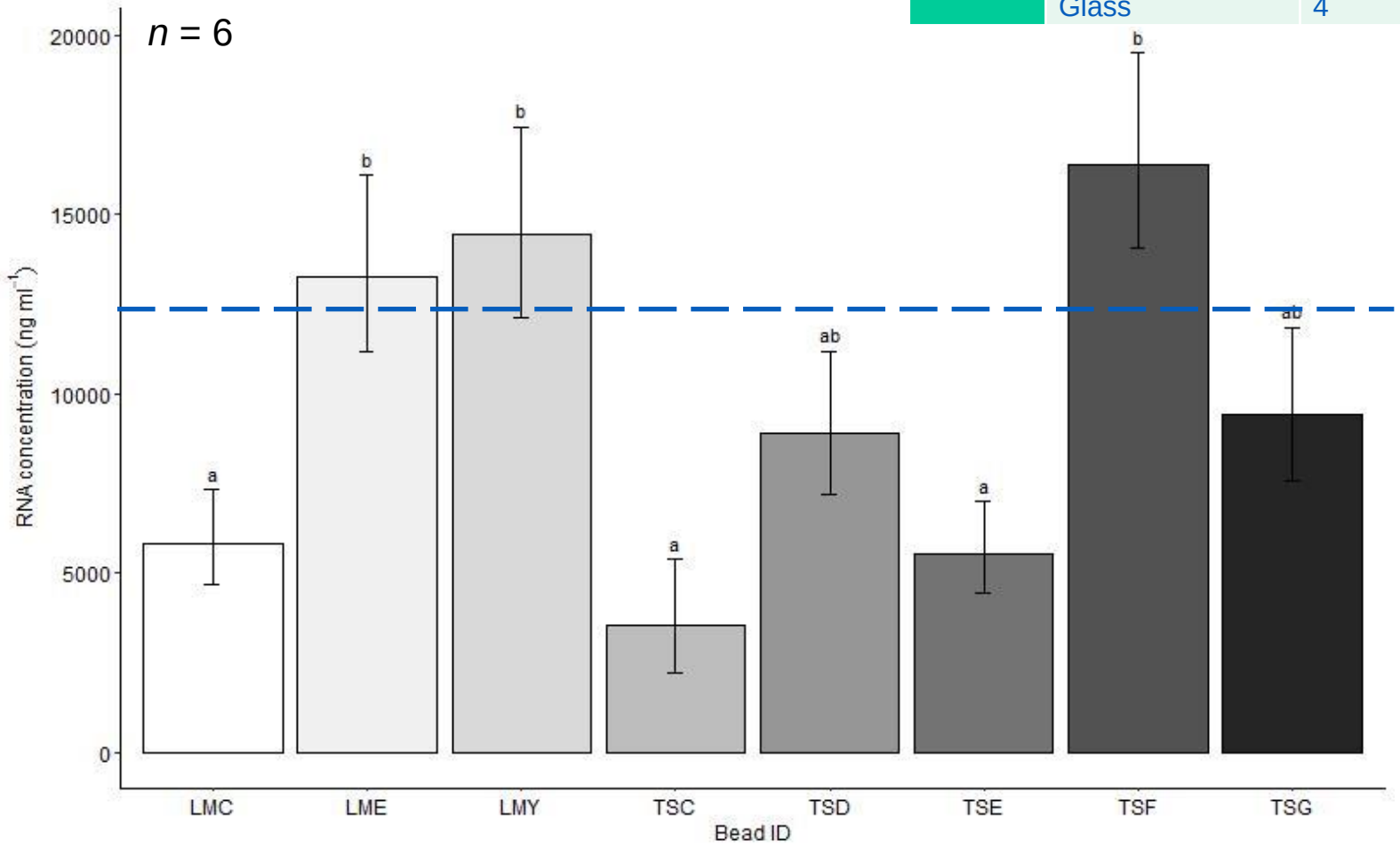


RNA extraction from pure culture



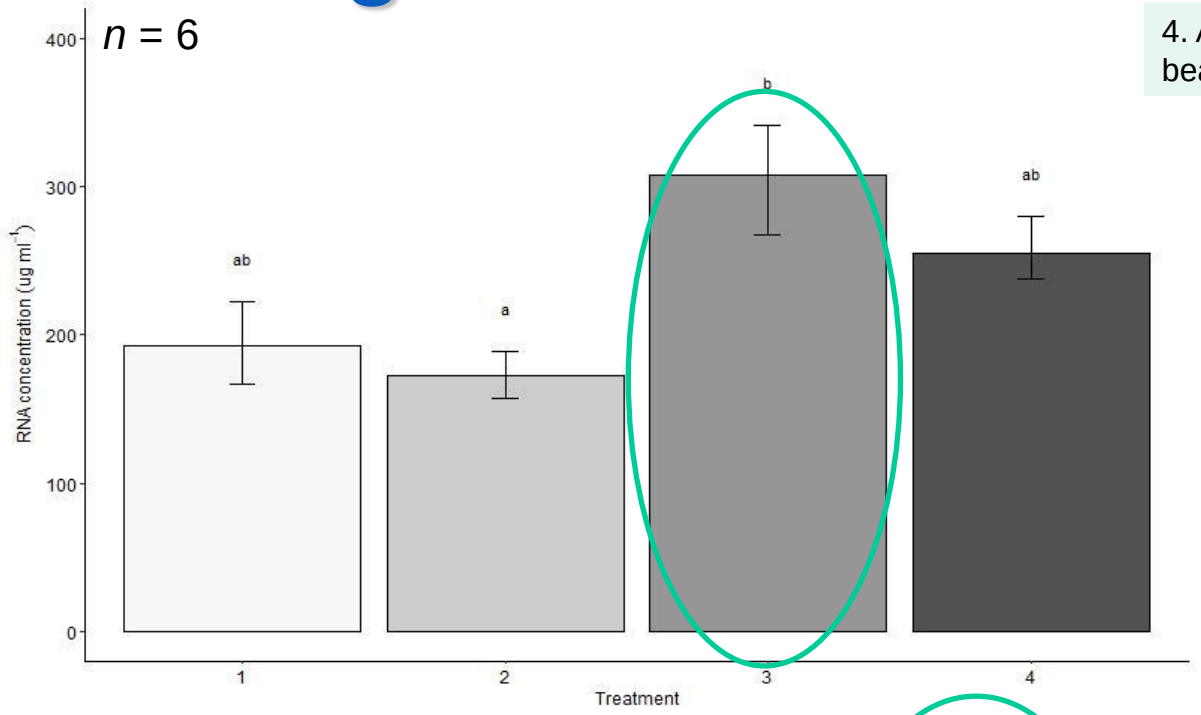
Optimising the assay: Bead size

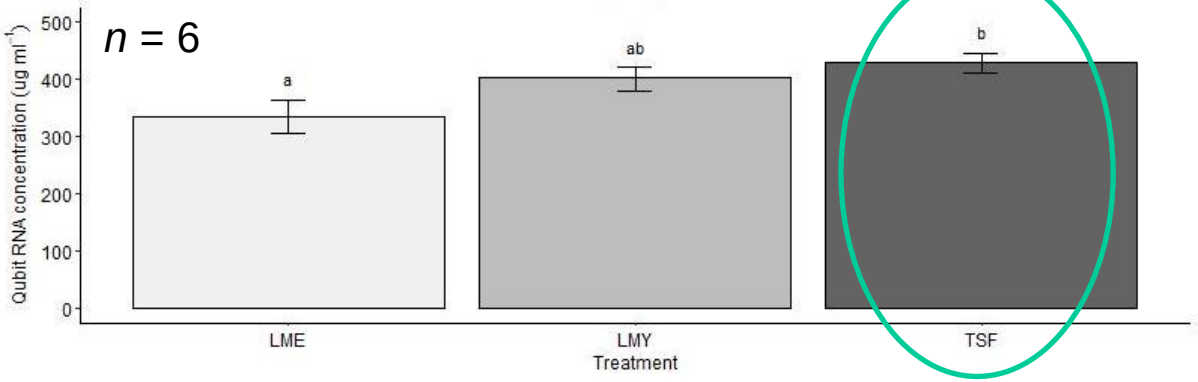
Bead ID	Material	Size (mm)
LMC	Silica	1.0
TSC	Glass	1.0
TSD	Zirconia/silica	1.0
LMY	Zirconium oxide	0.5
TSE	Glass	0.5
TSF	Zirconia/silica	0.1
TSG	Glass	0.1
LME	Ceramic	1.4
	Silica	0.1
	Glass	4



Optimising the assay: Beating times

Beating time
1. 40 sec @ setting 6.5
2. 25 sec, 5 sec break, 25 sec @ 6.5
3. 1 min, 2 min on ice, 1 min @ 6.0
4. As 3, but frozen for 30 min prior to beating





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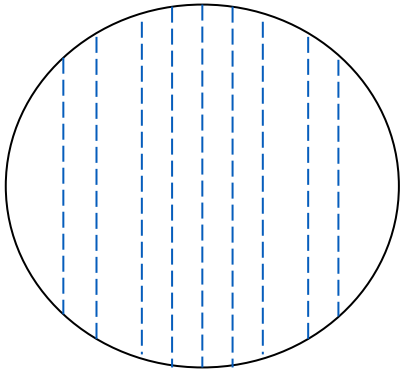
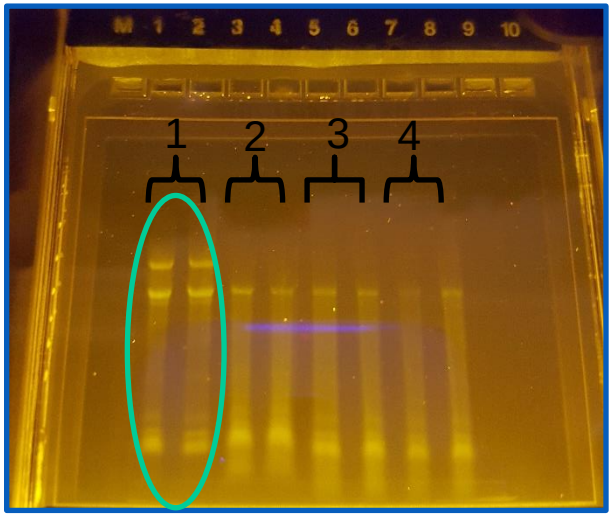
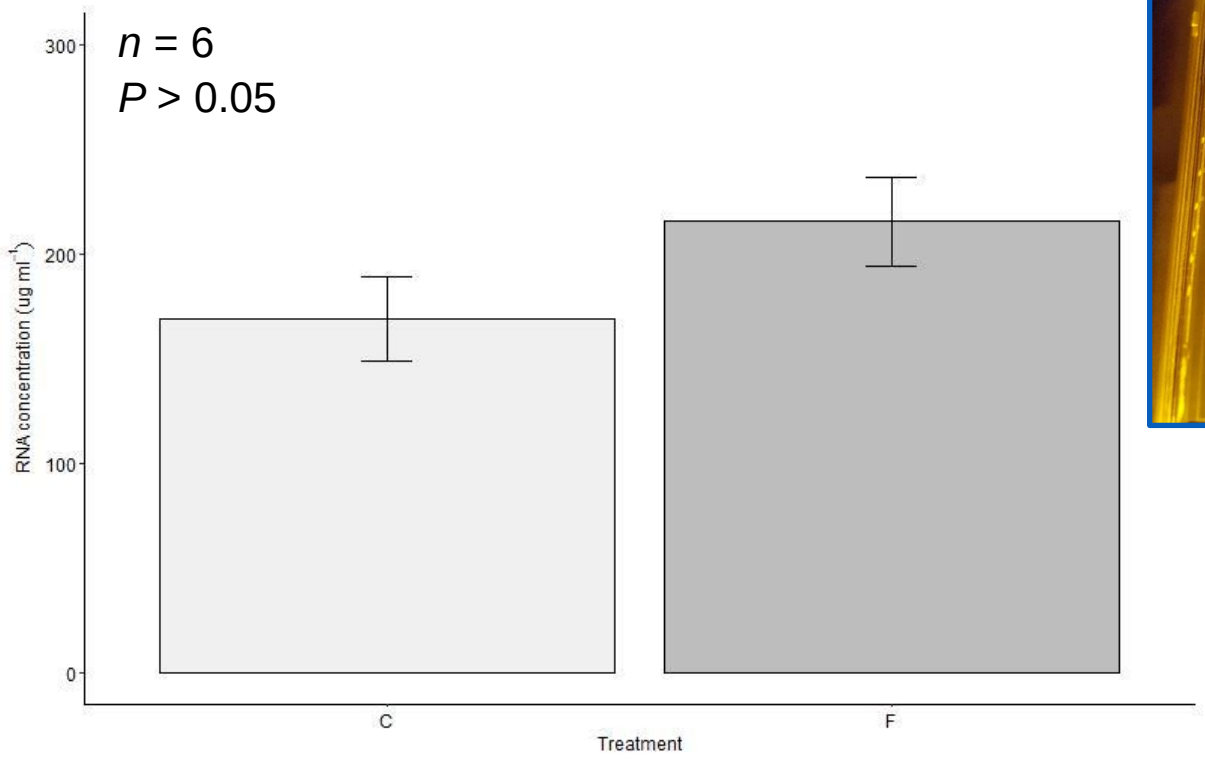
Optimising the assay: filters



25 mm, 0.8 µm polycarbonate filter

Beating time
1. 1 min, 2 min on ice, 1 min @ 6.0
2. As above for a total of 5 min
3. 5 min continuous @ 6.0
4. 5 min continuous @ 4.0





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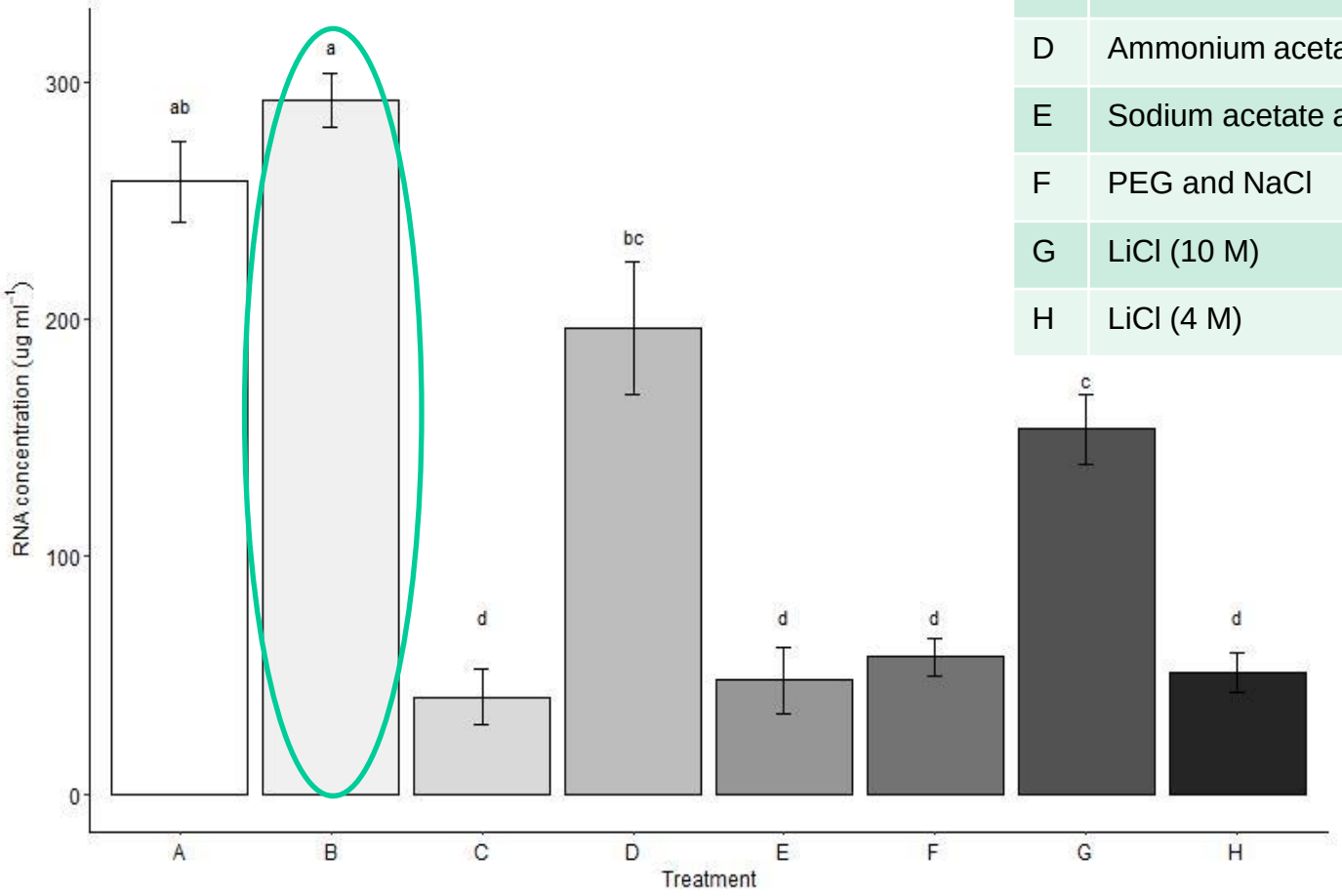
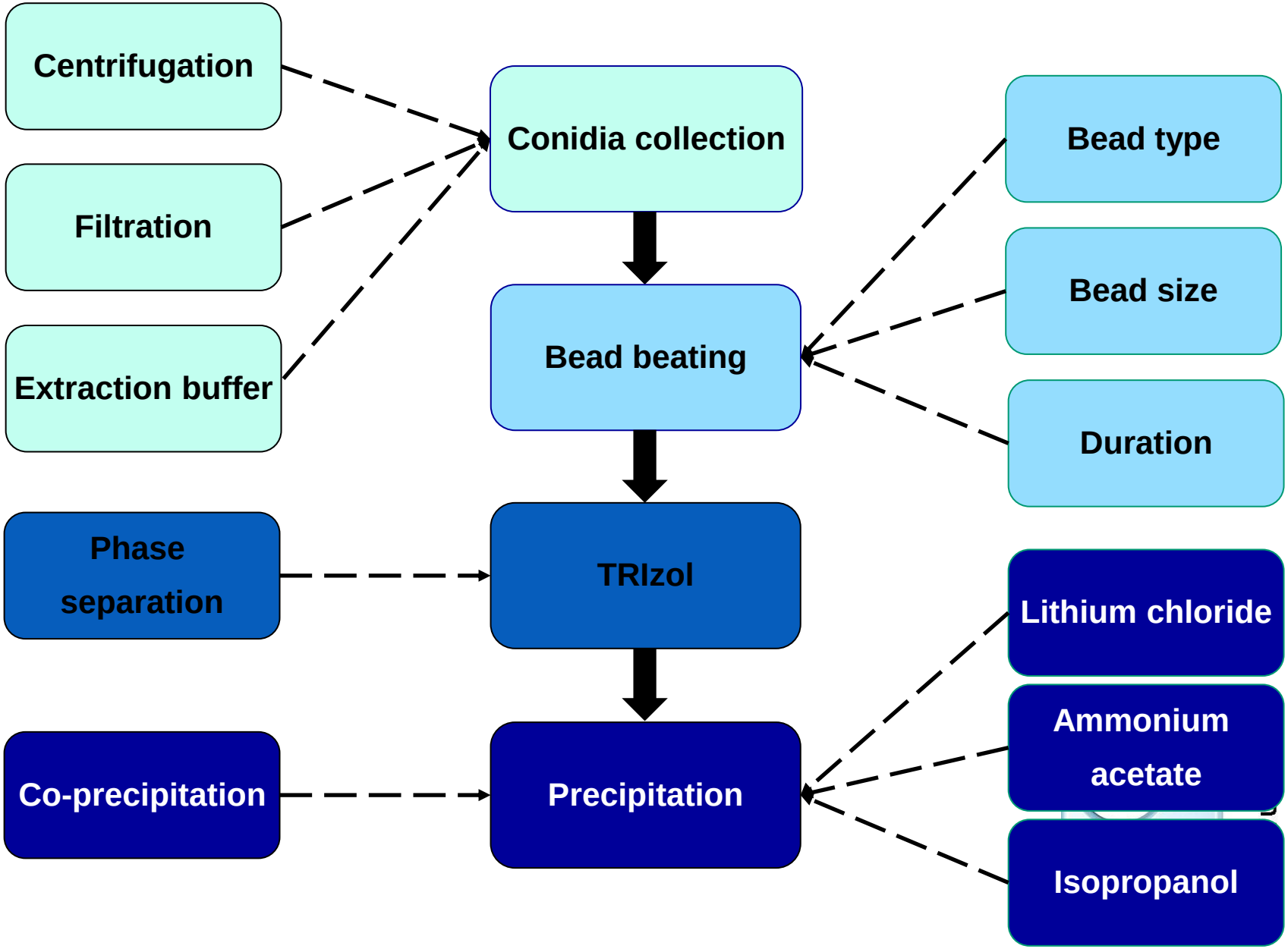
Optimising the assay: precipitation

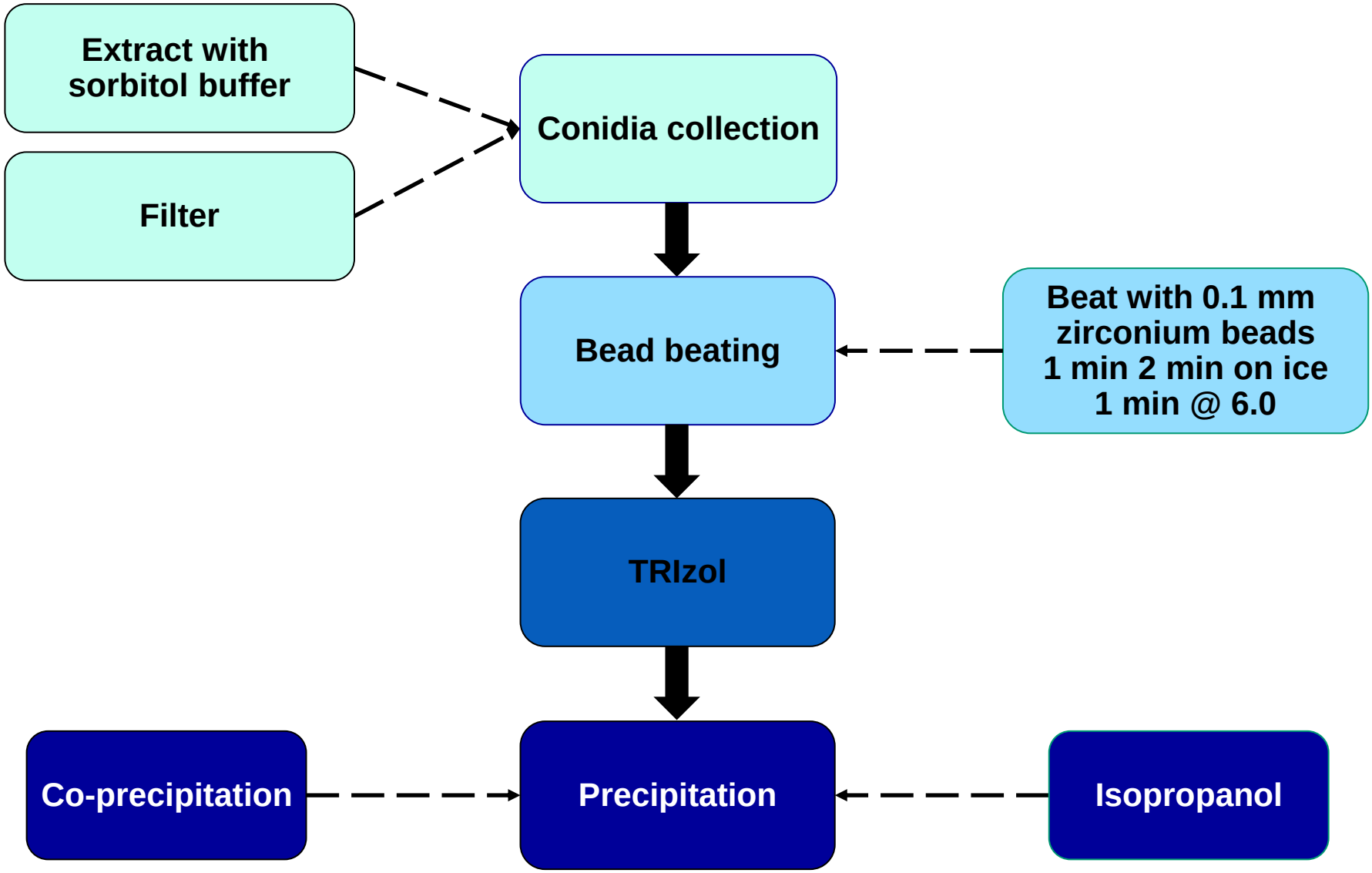
	Precipitation method
A	Isopropanol (12,000*g*, 10 min)
B	Isopropanol (12,000*g*, 60 min)
C	NaCl and isopropanol
D	Ammonium acetate and isopropanol
E	Sodium acetate and ethanol
F	PEG and NaCl
G	LiCl (10 M)
H	LiCl (4 M)



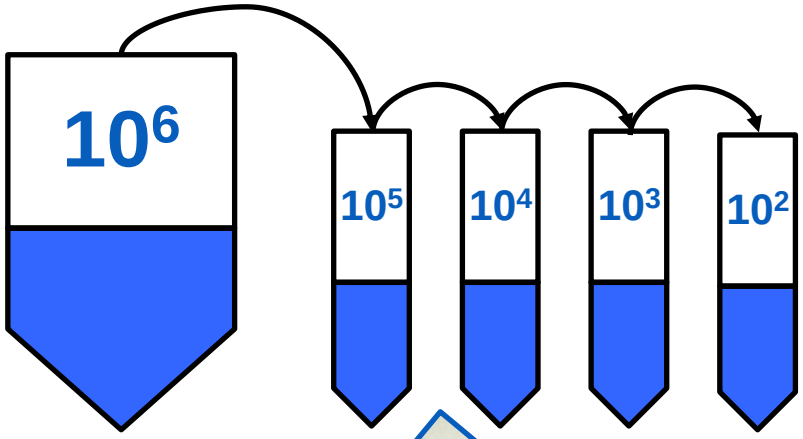
RNA extraction



RNA extraction



RNA Extraction: What next?



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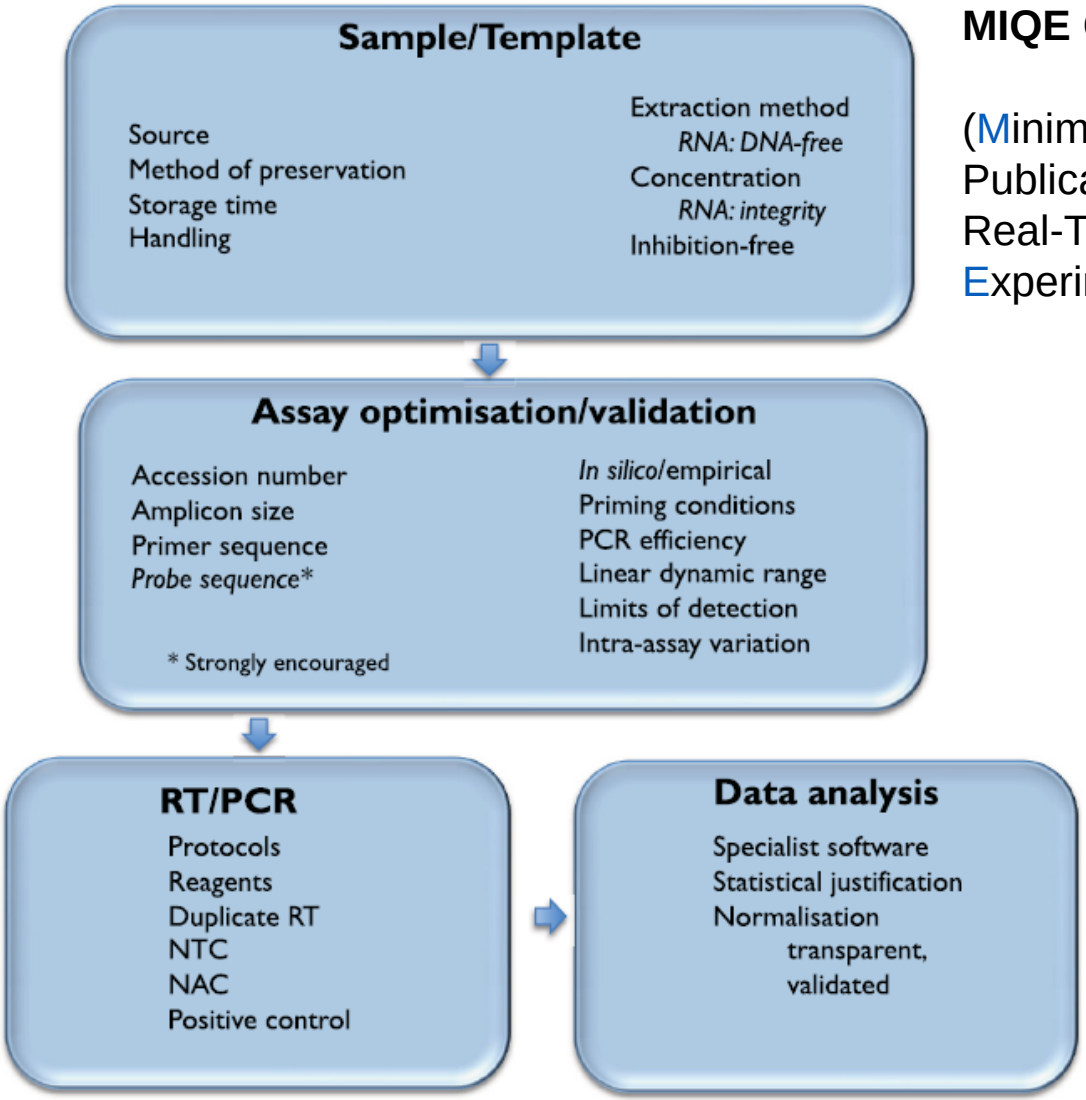
RT-qPCR assay



MIQE Guidelines

(Minimum Information for Publication of Quantitative Real-Time PCR Experiments)

Figure 1 Key criteria delineating essential technical information required for the assessment of a RT-qPCR experiment .

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Gene of interest

FKS1 gene

- Codes for the fksP protein
- Involved in the production of glucan in the cell wall
- Single copy gene

ACACGCTTCTTCTTGGATTGGATTTTGTCGCCTCTCTCGTACTCGGATCACTGGTTACAAGCGCAAGCTTCTTGGTGTGCCATCCGAGAAG
GGCTCCGGCGATGTTCCTAGGGCTCGTCTCACCAATATTTTCTTCAGCGAGATTATTGCACCCCTAGTACTTGTTGCTGTGACGCTGGTTCC
TTATCTCTACATCAACTCCAGGACTGGAGTACGTGATAACCCGGAACCTACGGATGCCATCCTGCGACTCGCCATTGTTGCCGCTGGACCAA
TCGCCATCAACGCAGGTGTTGCTGGTGTTTTCTTCGGCATGGCCTGCTGTATGGGACCCATCTTCAGCATGTGCTGCAAGAAATTCGGTGC
TGTTCTCGCTGCCATTGCGCACGCTATAGCTGTCATTGTACTGCTCGCCATCTTCGAAGTCATGTTCTTCCTTGAATCCTGGTCCTGGCCGAG
AATGCTCATTGGCATGATTGCAGCAGCGGCCATTCAGCGTTTCATCTACAAGCTCATCATTGCCCTTGCATTGACCCGCGAGTTCAAGCATG
ACCAGTCTAATATCGCATGGTGGACTGGCAAGTGGTACAACATGGGTTGGCACTCGATGTCACAACCCGGTCGTGAGTTCCTGTGCAAGA
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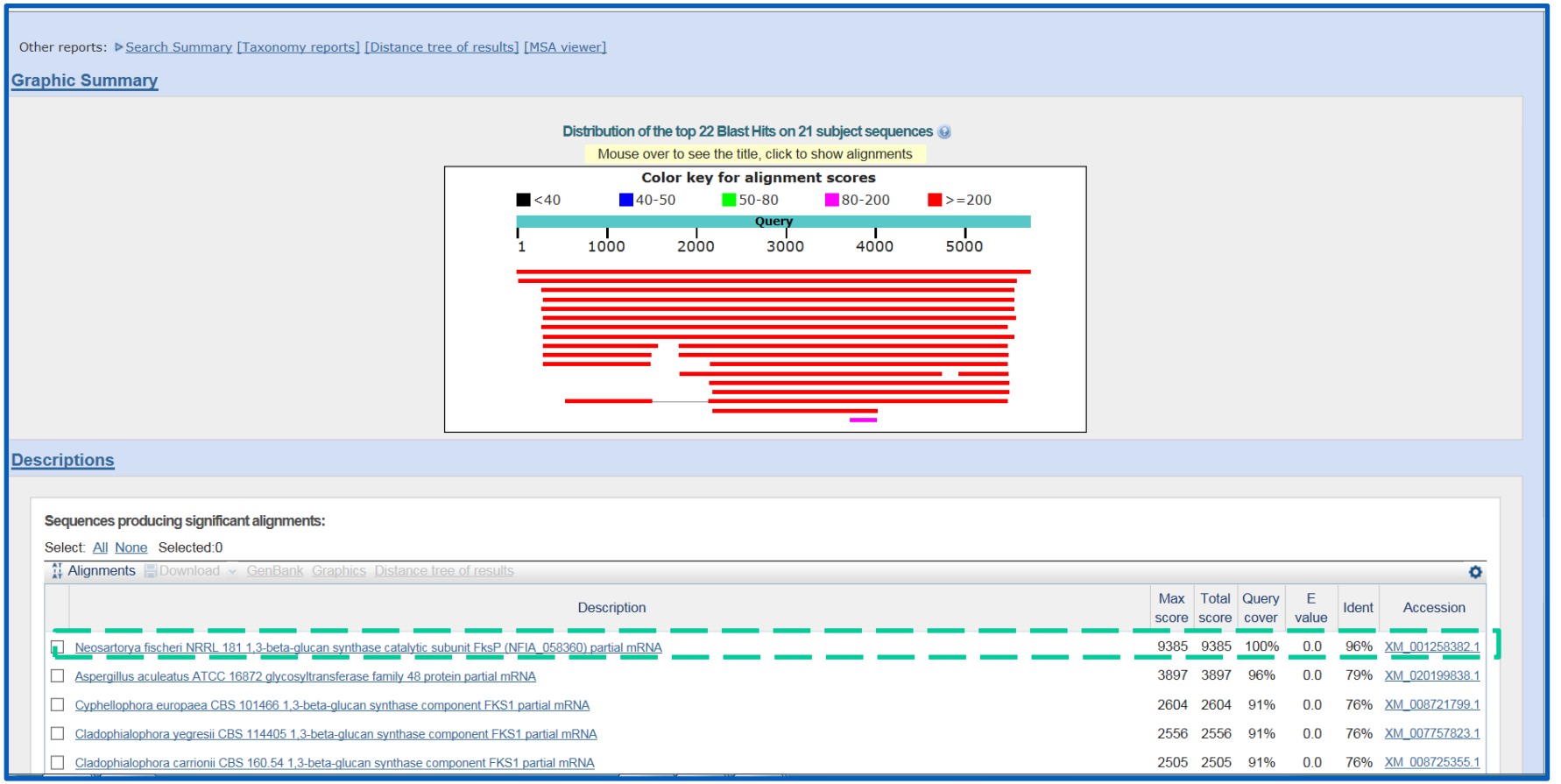
AATTCCACTCGGTCATGCTCTTCTGGCTGCGCCCAAGGTAAGGAACAATTTTCACCACGATATTTGATCTTATCTTGTCTAACACTCGTCTAG
TCGCCAAATCCGCCCTCCAATTTACTCACTGAAGCAATCGAAGCTCAGGAAGAGAAGAGTCATCCGCTTTGCTATTCTCTACTTTGGGATG
CTCATTCTGTTCCTTGTCCTTCTCATTGCCCCACTTGTTGTTCGCTCTATGGGATTGGTTAAAACTCCCAATCTTCCATTCAACTTGCTCCAGC
CTCTTGACAAGGACAACAACGATACTATGGTGACATACACCGGCAACAACATTCCCGCTGGATTTGAGCCAGTTGAGAGCGCCTCGAGCG
TGGCTACTGCAACGAGCTAG

Primer specificity

NCBI Blastn database



Primer specificity

Species	Strain	Accession no.
Aspergillus fumigatus	Af293	XM_746025.1
Aspergillus clavatus	NRRL 1	XM_001268294.1
Aspergillus flavus	NRRL 3357	XM_002383365.1
Aspergillus nidulans	FGSC A4	XM_656241.1
Aspergillus niger	CBS 513.88	XM_001390940.2
Aspergillus nominus	NRRL 13137	XM_015556015.1
Aspergillus oryzae	RIB40	XM_001816621.2
Aspergillus terreus	NIH 2624	XM_001212456.1
Baudoinia panamericana	UAMH 10762	XM_007675218.1
*Candida dubliniensis**	CD36	XM_002417149.1
Capronia coronate	CBS 617.96	XM_007727032.1
Cladophialophora bantiana	CBS 173.52	XM_016768205.1
Cladophialophora carrionii	CBS 160.54	XM_008725355.1
Cladophialophora immunda	Unknown	XM_016395370.1
Cladophialophora yegresii	CBS 114405	XM_007757823.1
Cyphellophora europea	CBS 101466	XM_008721799.1
Exophiala oligosperma	Unknown	XM_016405660.1
Exophiala spinifera	Unknown	XM_016384822.1
Fonsecaea erecta	Unknown	XM_018837737.1
Fonsecaea pedrosoi	CBS 160.54	XM_013423062.1
Neosartorya fischeri	NRRL 181	XM_001258382.1
Neurospora tetrasperma	FGSC 2508	XM_009854667.1
Penicillium chrysogenum Wisconsin	54-1255	XM_002564403.1
Penicillium digitatum	Pd1	XM_014679162.1
Saccharomyces cerevisiae	S288C	NM_001182231.1
Talaromyces atroroseus	Unknown	XM_020260475.1
Talaromyces marneffei	ATCC 18224	XM_002147459.1
Talaormyces stipitatus	ATCC 10500	XM_002481595.1
Trichoderma gamsii	Unknown	XM_018810875.1
Ustilago maydis	521	XM_011389324.1
*Verticillium albo-atrum**	VaMs.102	XM_003001921.1

In silico specificity:
Sequence is specific for *A. fumigatus*

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Primer specificity

Species	Strain
Aspergillus fumigatus	Af293
Aspergillus alliaceus (Petromyces)	DSMZ 813
Aspergillus clavatus	DSMZ 816 (NRRL 1)
Aspergillus flavus	DSMZ 1959
Aspergillus nidulans (Emericella)	DSMZ 820
Aspergillus niger	DSMZ 2143
Aspergillus terreus	DSMZ 826
Neosartorya fischeri	DSMZ 3700 (NRRL 181)
Penicillium glabrum	DSMZ 2017

In vivo specificity:

Awaiting results

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RT-qPCR assay: What next?

- Test the primers against microorganisms similar to *A. fumigatus*

- Test assay against *A. fumigatus* isolates that have been collected from field samples

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And finally…

Air sampling + RNA extraction+ RT-qPCR assay = Improved method of *A. fumigatus* detection

Expand the methods to include other pathogens of interest as part of our commercial Pathogen Suite

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Any Questions?

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