Nucleic acid diagnostic tests for the detection of microbial pathogens

Justin O' Grady[1], Ciara Mullen[1], Marcin Jankiewicz[1], Katrina Lacey[1], Nina L. Tuite[1], Louise O' Connor[1], Majella Maher[1], Thomas Barry[2], and Terry J. Smith[1]

[1] Molecular Diagnostic Research Group, National Centre for Biomedical Engineering Science, National University of Ireland, Galway, Ireland.

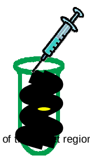
[2] Molecular Diagnostics Research Laboratory, Department of Microbiology, National University of Ireland, Galway, Ireland.

Abstract

The Molecular Diagnostics Research Group (MDRG) at NUI Galway has over 20 years experience in the field of molecular diagnostics. Research activities include molecular diagnostic target discovery and nucleic acid based test design and development. Over the years the group has identified a number of platform nucleic acid diagnostic test (NAT) target technologies for the sensitive and specific detection and identification of bacterial and fungal pathogens. The NAT target technologies have been demonstrated in a wide range of test formats including, real-time PCR, real-time NASBA and direct nucleic acid target detection systems. Applications of the NAT technology include clinical, food, veterinary and environmental sectors. Successful commercialisation of our nucleic acid targets has occurred through licensing agreements and research and development collaborations with many of the major international diagnostics companies. Detailed below are some of the strategies and methodologies employed by the MDRG in the design and development of NATs for the detection and identification of micro-organisms.

Step 1: Target discovery

The main activities of the MDRG include molecular target identification and design & development of nucleic acid based diagnostic tests. A number of platform nucleic acid diagnostic test (NAT) targets, for sensitive and specific identification of bacterial and fungal pathogens have been developed by the MDRG. These targets have been used in a number of NAT formats for application in the clinical, food, veterinary and environmental sectors (1-9). The group has collaborated with major IVD companies, developing assays for infectious diseases.



Criteria for a suitable diagnostic target

- A well-characterised, variable nucleic acid sequence.
- Must be present in all micro-organisms of interest.
- Should exhibit 5' and 3' flanking sequence conservation (enables universal amplification of t… region)
- Should exhibit internal sequence variability (enables the design of genus and species-specific NAD assays)
- Where necessary, should code for a high copy number RNA target (might provide increased assay sensitivity)

Step 2: Target sequencing strategy

In order to ensure target specificity, an initial sequencing program is developed which involves sequencing of the target DNA from a number of geographically distinct strains of each of the species of interest. It is also important to generate sequence information for closely related species and species which are common to the target sample environment. No recommendation on the number of organisms on a specificity panel can be made as it depends on the target organism, the application of the assay and the sample environment. The MDRG offers the following recommendations regarding the number of strains of each species on the specificity panel that should be sequenced (Table1):

Table 1: Recommendations for number of strains of each species to be included on a specificity panel and sequenced

	No. of strains of each species included in sequencing and specificity panels.
Target species	10-20
Genus related species	5-10
Phylogenetically related species	2-5
Species related to sample environment	1-3

Step 3: Sequence analysis and assay design

In silico analysis of all generated sequences is then performed. Areas of high sequence variability between target and non-target organisms are chosen for assay design.

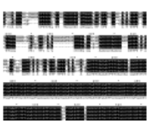


Fig. 1: Sample sequence alignment.

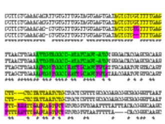


Fig. 2: Alignment showing an example of a potential TaqMan probe assay. Primers are shown in yellow, probe in green. Mis-matches are highlighted in pink.

Step 4: Assay Performance Evaluation and Optimisation

The performance of the assay is evaluated with respect to specificity and sensitivity.

Specificity: Inclusivity and Exclusivity of the assay is assessed.

Sensitivity: The LOD of the assay is determined (ideally 1-10 cell equivalents)

Optimisation: Reaction reagents and cycling conditions are optimised.

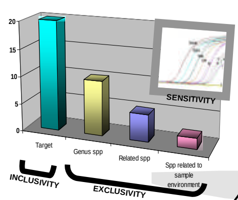


Fig. 3: Assay performance evaluation

Example 1: *RiboSEQ* NAT for *Listeria monocytogenes*

- A real-time PCR method, based on FRET hybridization probe technology (HybProbe), was developed to specifically detect the *ssrA* gene of *L. monocytogenes* and demonstrated on a LightCycler® instrument (1 & 2).
- The specificity of the test was confirmed against a panel of 27 *L. monocytogenes* strains, 14 *L. innocua* strains, 4 other *Listeria* species and 26 closely related bacteria (Fig. 4) (1 & 2).
- The sensitivity of the test was determined using ten-fold serial dilutions of *L. monocytogenes* genomic DNA. The detection limit was 1-10 *L. monocytogenes* cell equivalents (Fig. 5)(2). Artificially contaminated food samples (spiked with ~ 1 CFU) were then demonstrated positive for the presence of *L. monocytogenes* (Fig. 6) (2).
- In a further study, 175 natural food samples were tested with the assay and results compared to those obtained using the ISO 11290-1 standard method (1). The rapid method was 99.44% specific, 96.15% sensitive and 99.03% accurate using the culture method as the gold standard.
- The test enables *L. monocytogenes* to be identified in foods in 2 working days compared to 5-7 for standard microbiological methods (1 & 2).

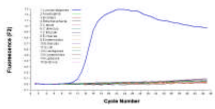


Fig. 4: Example of a quantification curve demonstrating the specificity of the *L. monocytogenes* real-time PCR test

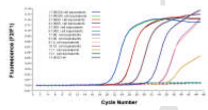


Fig. 5: Sensitivity of the *L. monocytogenes* real-time PCR test using serial ten-fold dilutions of *L. monocytogenes* genomic DNA

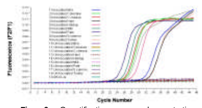


Fig. 6: Quantification curve demonstrating detection of *L. monocytogenes* in 9 inoculated foods in the *L. monocytogenes* real-time PCR test

Example 2: *RiboSEQ* NAT for *Group B Streptococcus*

- The RiboSEQ GBS real-time PCR test targets the bacterial *ssrA* gene and includes a GBS-specific hybridisation probe. The test is performed on the Roche LightCycler® instrument (9).
- Specificity studies were performed using a panel of 10 GBS species representing the 7 most commonly occurring serotypes and 42 related streptococci and other species found in the genital tract environment (Figure 7).
- Sensitivity of detection was established using DNA extracted from serial dilutions of GBS culture (10^4–10^{-1} cells) (Figure 8).
- An internal amplification control consisting of heterologous DNA cloned into a plasmid vector was included in the test to identify false negative test results caused by PCR inhibition (Figure 9).

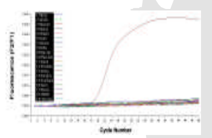


Fig 7: Real-time PCR quantification curve for the *RiboSEQ* GBS-specific test with GBS (brown line) and 17 non-GBS species

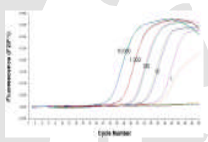


Fig 8: Quantification curve for the *RiboSEQ* GBS real-time PCR test using DNA template equivalent to 10,000 - 0.1 cells.

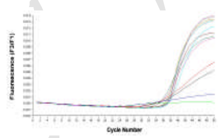


Fig 9: Quantification curve for internal control target included in the *RiboSEQ* GBS real-time PCR test shown in Figure 8.

- The performance of the RiboSEQ GBS real-time PCR test was evaluated in 159 clinical samples and was benchmarked against the performance of the commercially available FDA-approved BD GeneOhm™ StrepB Assay. The results from both tests were correlated with the results of microbiological culture of the samples (LIM broth and TSA + 5% sheep blood agar) (Table 2)
- In this study, the RiboSEQ GBS test performed better than the commercial BD GeneOhm™ StrepB test
- The RiboSEQ GBS test is a valuable method for the rapid, sensitive and specific detection of GBS in pregnant women. It has potential for application in the clinical setting with the capability of identifying GBS in a test time of approximately 75 min (9).

Table 2: Summary of clinical evaluation

		RiboSEQ GBS test		BD GeneOhm™ StrepB Assay	
		Positive	Negative	Positive	Negative
Culture[a]	Positive	107	4	105	6
	Negative	2	46	5	43
	Sensitivity 95%CI	96.4% (90.5–98.8%)		94.6% (88.1–97.8%)	
	Specificity 95%CI	95.8% (84.6-99.3%)		89.6% (76.6–96.1%)	

References

1. **O'Grady, J., Ruttledge, M., Sedano-Balbas, S., Smith, T., Barry, T., Maher, M.** 2009. Rapid detection of Listeria monocytogenes in food using culture enrichment combined with real-time PCR. Food Microbiol.
2. **O'Grady, J., Sedano-Balbas, S., Maher, M., Smith, T., Barry, T.,** 2008. Rapid real-time PCR detection of Listeria monocytogenes in enriched food samples based on the ssrA gene, a novel diagnostic target. Food Microbiol. 25, 75–84.
3. **Kavanagh, S., Brennan, C., O' Connor, L., Moran, S., Salas R., Lyons, J., Silke, J. and Maher. M.** (2008). Real-time PCR detection of *Dinophysis* species in Irish coastal waters. *J. Marine Systems*. (In press)
4. **Glynn B., Lacey, K., Reilly, J., Barry, T., Smith, T. and Maher, M.** (2007). Quantification of bacterial tmRNA using *in vitro* transcribed RNA standards and two-Step qRT-PCR. *Res. J. Biol. Sci.* 2, 564-570.
5. **Maher, M., Kavanagh, S., Brennan, C., Moran, S., Salas, R., Lyons, J. and Silke J.** (2007). Nucleic acid tests for toxic phytoplankton in Irish waters- *Phytotest*. Proceedings of the 7th Irish Shellfish Safety Workshop, Galway November 2006. Marine Environment and Health Series, No 27, 2007 pp.70-77.
6. **Glynn, B., Lahiff, S., Wernecke, M., Barry, T., Smith, T. and Maher, M.** (2006). Current and emerging molecular diagnostic technologies applicable to bacterial food safety. *Int. J. Dairy Technol.* 59, 126-139.
7. **O' Connor, L., Lahiff, S., Casey, F., Glennon, M., Cormican, M. and Maher, M.** (2005). Quantification of ALS1 gene expression in *Candida albicans* biofilms by RT-PCR using hybridization probes on the LightCyclerTM. *Mol. Cell. Probes*. 11, 153-162.
8. **Fitzmaurice, J., Glennon, M., Duffy, G., Sheridan, J., Carroll, C. and Maher, M.** (2004). Application of real-time PCR and RT-PCR assays for the detection and quantitation of VT1 and VT2 toxin genes in *E. coli* 0157:H7. *Mol. Cell. Probes* 18, 123-132.
9. **Wernecke M., Mullen C., Sharma V., Morrison J., Barry T., Maher M. and Smith T. Evaluation of a novel real-time PCR test based on the *ssrA* gene for the identification of group B streptococci in pregnant women. Submitted to BMC Infectious Diseases.**