Amplification-free sensing of circulating nucleic acid biomarkers in blood:
A minimally invasive tool for early diagnosis of cancer
Molecular Bioengineering

How an academically trained chemist (or molecular bioengineer) working in a Bioengineering department can address public health problems and engineer solutions for cancer
Our goal: Diagnose more cancers better and earlier

Increasing survival rate by treating early, only if necessary and with the best suited drugs requires...

- Highly specific biomarkers (or combination of biomarkers)
- Non-invasive tests for widespread public screening (affordable, robust and easy-to-use)
Prostate cancer: A case study

Prostate cancer is currently diagnosed too late

In the UK only, around 35,000 new cases diagnosed per year, around a quarter would be already metastatic - but that's only in the UK.

And non-invasive diagnostic blood tests lack specificity

Current diagnosis results from the presentation of clinical symptoms such as urine hesitancy, erectile dysfunction and decreased urination flow, whereupon a plasma or serum sample’s prostate specific antigen (PSA) concentration will be assessed. Typically, results of 4ng/ml or above result in a prostate biopsy to confirm a neoplasm. However, it is reported that over 75% of positive PSA tests are in fact false positives.
There is currently no screening programme for prostate cancer in the UK [...] Instead of a national screening programme, there is an informed choice programme on prostate cancer risk management. It aims to give men good information on the pros and cons of a PSA test.

Private healthcare offers patients a prostate cancer screening service.

ProstateCheck: £360
Follow-up appointments range from £200 to £250
Could eliminate 30-50% of all unnecessary biopsies
Our answers to an unsolved problem

- Minimal sample volume
- Automated sample processing
- Benchtop analysis
- High specificity
- Low cost
Our Target: Circulating micro RNAs

Need for reliable, highly sensitive, specific and quantitative sensing technology

• Secretion in body fluids (saliva, serum, plasma...)

Ideal for minimally invasive diagnosis

BUT

• Low concentration in these cell-free fluids

• miRNAs have very similar sequences
The gold standard: **RT-qPCR and Taqman probes**

- Taqman probes are expensive and often unreliable (lack of specificity, reproducibility, and strong background fluorescence).
- Double amplification (prone to contamination and source of errors).
Our Strategy: Oligonucleotide-templated reactions

Using a DNA or RNA strand as a template to catalyse an otherwise unfavourable reaction of formation of a fluorescent dye

Our scaffold: Peptide Nucleic Acids (PNAs)
Our probe-heads: A toolbox of multicolour dyes

Possibility of multiplexed (multicolour) analysis

Our Chemistry: Reaction of fluorescence unquenching

Quenched fluorescence (by PET)

PNA\textsubscript{1} DNA or RNA template PNA\textsubscript{2}

Strong Fluorescence

Emission $\lambda_{\text{max}} = 520-530$ nm

Proof-of-concept study

MiR-141 and miR-375: biomarkers for prostate cancer (high levels compared to healthy controls)

MiR-132: biomarker for ovarian cancer (low levels compared to healthy controls)
In vitro validation

Quantitative detection
Relatively fast

Specific detection

Specificity at the single nucleotide level (but position-dependent)

Implications for miR141 and miR375 sensing?

G. Metcalf, A. Shibakawa, H. Patel, A. Sita-Lumsden, A. Zivi, N. Rama, C.L. Bevan, S. Ladame
*Anal. Chem.*, 2016, accepted
**miR375**: No other miR with sequence homology >75%

**miR141**: 90.90% homology (20/22bp) with miR-200a

miR-141 sequence (5’ to 3’): UAA CAC UGU CUG GUA A
miR-200a sequence (5’ to 3’): UAA CAC UGU CUG GUA A

Likely to detect both miR141 and miR200a simultaneously
The issue of **Sensitivity**

*LITTLE and CONTRADICTORY* information on endogenous miRNA concentrations available

highly dependent on the sample has been processed and analysed

**PRACTICAL APPROACH:**

Is our technology FIT-FOR-PURPOSE?

- Human serum
- Total RNA extraction
- RT-qPCR
- PNA probes

Picomolar? Femtomolar? Attomolar?
**Validation on Pca samples**

- **RN**: Remission, post-prostate removal
- **RY**: Remission, prostate gland present
- **AL**: Localised advanced tumour
- **AM**: Metastatic advanced tumour

**miR-141**

- Technology
- fit-for-purpose

- Amplification-free Detection

- Good correlation with RT-qPCR data

**miR-375**

- PCa diagnosis and grading

**Cross-validation:** ovarian vs prostate cancer

- Can detect both increasing and decreasing levels of miRNAs
- miR141 behaves as reference miRNA for ovarian Cancer
Why no amplification required?

Mature vs pre-miR

PNA probes can detect mature miRNAs but also their precursors
Clot blood – leave upright at RT (0.5-1h)  
Collect supernatant - aspirate  
Aliquote (1ml/cryovial)  
Freeze at -80°C  
Add TRIzol® and chloroform to serum (5min)  
Precipitate RNA from supernatant with isopropanol (1min)  
Wash three times with 75% ethanol  
Resuspend RNA in nuclease-free ddH₂O  
Heat shock 55-60°C (10min)
Can we avoid RNA extraction?
Vision for the future

• A technology of broad applicability, also to cancers with low survival rates (lung, pancreatic, oesophageal)

• A versatile technology suitable for the detection of any circulating nucleic acid biomarkers (combining miRNA, cell-free DNA and SNP detection in one device for improved specificity).

  • Minimally invasive (simple blood test)
  • Highly automated (minimal sample processing)
  • Low production cost (probes costing <1p per test)
  • Amenable to incorporation in portable devices (isothermal, no enzyme, fast)
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