BioMed Central

Research

Application of *in situ* reverse trancriptase-polymerase chain reaction (RT-PCR) to tissue microarrays

Alasdair C Stamps*, Jonathan A Terrett and Paul J Adam

Address: Oxford GlycoSciences (UK) Ltd., The Form, 86 Milton Park, Abingdon, UK OX14 4RY

Email: Alasdair C Stamps* - astamps@ntlworld.com; Jonathan A Terrett - jon.terrett@ogs.co.uk; Paul J Adam - paul.adam@ogs.co.uk

* Corresponding author

Abstract

Detection of disease-associated gene transcripts in primary disease tissues is frequently confounded by the presence of non-involved cell types. Alternative methods of detecting gene expression directly within tissues involve either the generation of antibodies, which can be a lengthy process and may suffer from lack of specificity, or amplification of reverse-transcribed cDNA in tissue sections (*in situ* RT-PCR). The latter method is highly specific and enables detection of transcripts in the cells originally responsible for their synthesis, but is highly destructive of tissue structures and can be carried out on only one or a few sections per experiment, resulting in low reproducibility. In this study, *in situ* RT-PCR was applied for the first time to commercially available tissue section microarrays enabling the examination of up to 70 different samples simultaneously. Modifications to the technique are detailed that preserved visible tissue and cellular structures and improved transcript detection whilst preventing significant generation of artefacts.

Background

Prior to the advent of miniaturisation, the study of cellular gene expression took the form of either antibody-protein detection, such as Western blotting of protein lysates or immunohistochemistry (IHC) on tissue sections or immobilised tissue culture cells, or nucleic acid analyses such as Northern blot hybridisation of RNA transcripts or PCR amplification of reverse-transcribed cDNA. Miniaturisation technologies are now long established in the measurement of RNA expression levels, in the form of nucleic acid microarray. Compared to classical Northern blotting or even the small amounts of material required for PCR, the microarray presents significant economies of scale and can equal or better the specificity of other techniques, including the detection of single nucleotide polymorphisms and splice variants. High throughput is, however, expensive, and particularly in clinical studies may not be practical from the point of view of sample sizes. Primary

samples are also heterogeneous and although microarray and other techniques may reveal variations in gene expression, they do not identify the cell type responsible for the differential. Although the use of antibodies in IHC has the advantage of detecting *in situ* the protein product of gene expression, the origin of secreted proteins such as growth factors, cytokines and serum markers may not be revealed by this technique. Miniaturisation of protein detection has given rise to some notable technologies e.g. surface plasmon resonance and emerging 'protein chip' platforms. This area of development is less advanced than genomic microarray and faces greater challenges due to the less predictable nature of protein interactions in solution, and the greater difficulty in synthesising, and optimising conditions for detection molecules.

The specific amplification of cytosolic mRNA molecules in paraffin-embedded tissue sections was first reported by
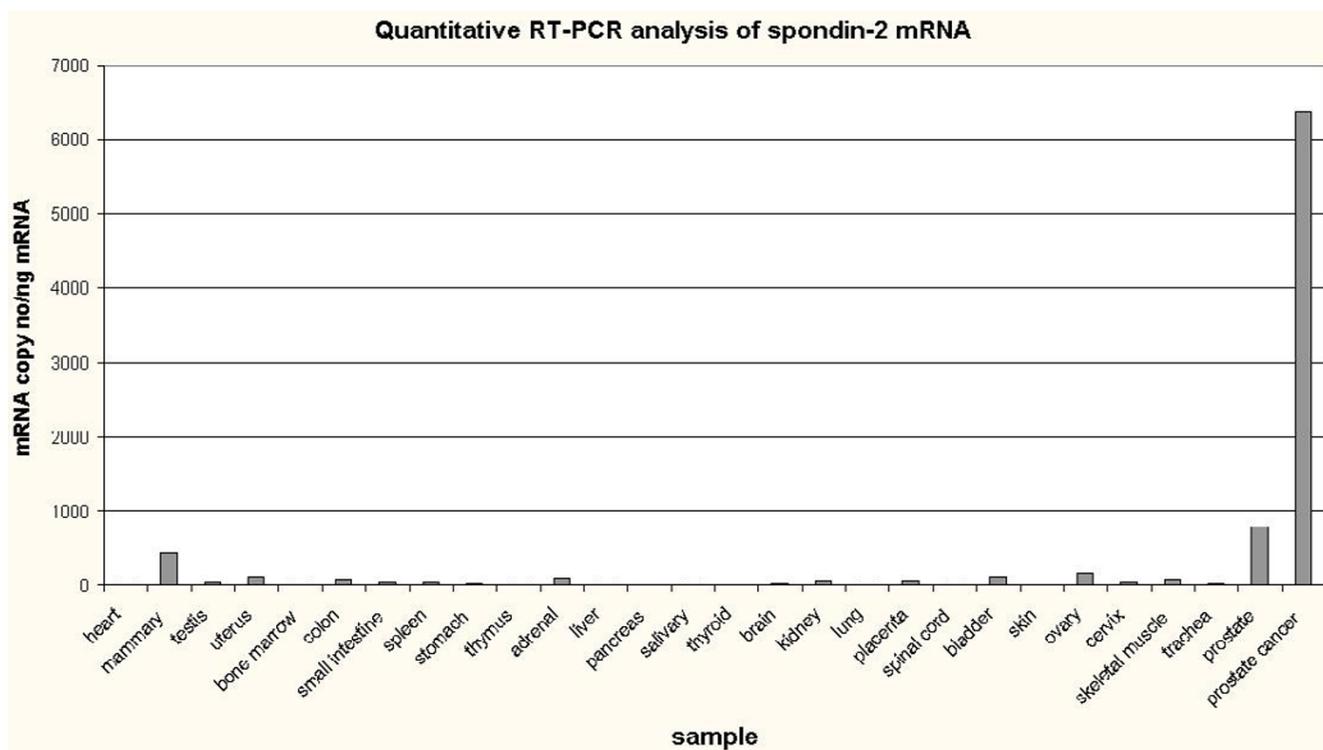
Figure 1
Quantitative RT-PCR analysis of human primary normal and cancer tissues. Amplification of reverse-transcribed mRNA was carried out on pools of normal human tissue as indicated and on a pool of prostate cancer samples, as described in Methods, and measured by SYBR-green staining and fluorescence quantification. Results were calculated relative to a set of standards and expressed here as copy number of original transcripts per ng of mRNA.

Staecker *et al.* [1], using fluorescence detection of amplified molecules. Use of the technique is not as widespread as other RT-PCR methods, however, and this may be due to technical complexity and an inherent lack of reproducibility [2,3]. The processing of large numbers of samples is also slow and sequential, adding to variability issues.

We present for the first time, the application of in situ RT-PCR to the detection of specific RNA transcripts in histological microarrays of up to 250 different samples per slide, using modifications of the procedure to maximise reliability and sensitivity. The modified method may be carried out entirely using commercially available materials, including tissue microarrays.

Results
Real-time quantitative RT-PCR analysis of expression of the extracellular matrix protein gene spondin 2 [4] was carried out on a series of total RNA preparations from normal human tissue and paired normal/cancer samples. Selection of sequences for oligonucleotide primers was carried out with the aim of amplifying sequences that

spanned two or more exons, such that the intervening introns in the corresponding genomic DNA would distance the two primers beyond the capacity of the PCR method to amplify. The DNA sequence of the spondin 2 gene was downloaded from Ensembl [5] and primer sequences selected from exon 4, nucleotides 3 to 25, and exon 6, nucleotides 14 to 36, in order to generate a 300 base pair (bp) PCR product from reverse transcribed spondin 2 cDNA. The same primer sites in the genomic DNA were separated by 3567 bp, preventing detectable amplification from this source. Significant elevation of expression was observed in prostate cancer compared to normal prostate tissue and all other normal tissues tested (Figure 1). In the same reactions, a pair of 'scrambled' primers, unrelated to any known DNA sequence, yielded no amplification products.

The same primers were used in *in situ* RT-PCR analysis of 1 mm paraffin-embedded tissue section arrays, as described in Materials and Methods (Figure 2). Experience with immunohistochemistry on tissue microarray showed that sections on poly-L-lysine coated miroscope slides
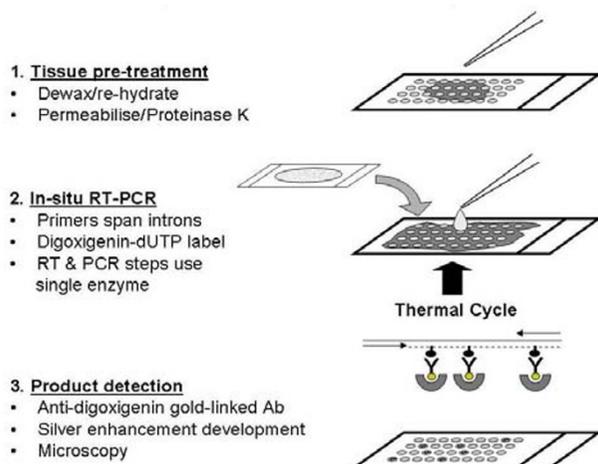
Figure 2
Flow chart describing the application of *in situ* RT-PCR to tissue microarrays. Step 1: Slide preparation. Sections were deparaffinated by xylene washes, and celluar material permeabilised by limited proteinase K digestion; Step 2: amplification of reverse-transcribed cDNA using intron-spanning PCR primers (horizontal arrows) added in a single mix of reverse transcriptase, rTth polymerase and deoxyribonucleotides spiked with digoxygenin-labelled dUTP (black ovals); Step 3: visualisation of PCR products by binding to digoxygenin-specific gold-labelled antibodies (yellow dots), followed by silver nucleation around the bound gold particles (grey crescents).

Figure 3
***In situ* RT-PCR analysis of tissue microarrays.** Slides containing up to 250 sections of 1 mm diameter, taken from prostate cancer biopsies, were subjected to *in situ* RT-PCR as described in Methods, using either spondin-2-specific oligonucleotide primers (i) or control 'scrambled' primers (ii). PCR prodcts were labelled by Immunogold with silver enhancement; after hematoxylin staining to highlight cell nuclei (n), sections were visualised by light microscopy at 40× magnification.

were less prone to detachment during processing, so these were chosen for the *in situ* RT-PCR experiments. Whilst the standard de-waxing step was carried out to remove the embedding material, the extensive deoxyribonuclease (DNAse) treatment step [6] was omitted in order to better preserve structural features on the tissue sections. The number of PCR cycles used was set at 20, within the linear range of amplification defined by real-time quantitative RT-PCR (data not shown). Depending on the density of sections on the slide, up to 70 could be covered by a single coverslip. Successful amplification was indicated by the presence of silver grains visible under light microscopy (Figure 3), whilst the hematoxylin counterstain highlighted cell nuclei to provide a necessary distinction from the cytoplasm, where all of the specific amplification occurred. It was clear from the pattern of silver grains that the increase in expression of spondin-2 at the transcriptional level was confined to the cancer cells themselves, which were distinguished by their large nuclei and disorganised morphology. In control experiments using 'scrambled' primers in the place of spondin-2 specific oligonucleotides, no amplification was observed.
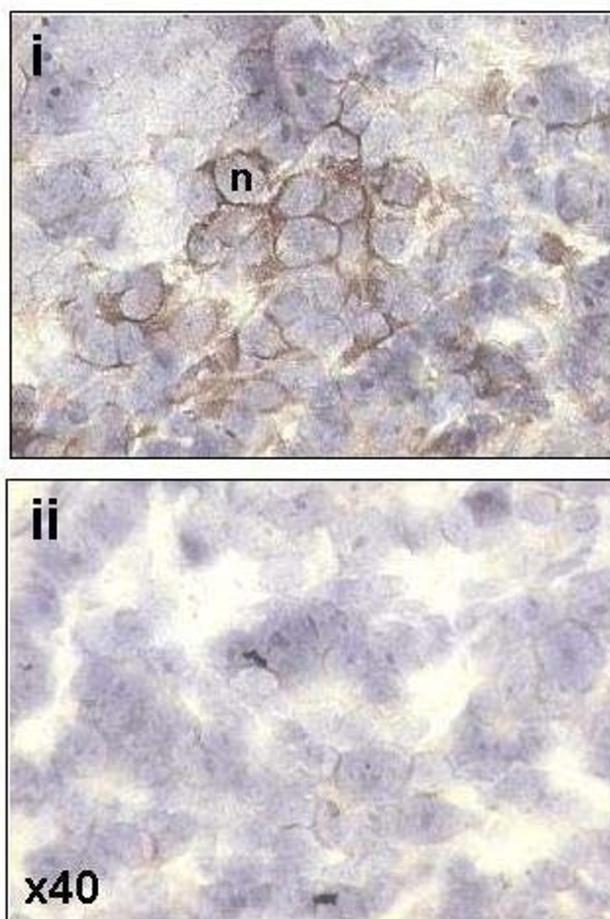
Discussion

The use of in situ RT-PCR to examine gene expression in disease tissues has certain advantages over more established hybridisation, PCR amplification or antibody-based techniques. As with immunohistochemistry, detection of gene expression is at the level of individual cells [7], but whereas polyclonal antibody production by immunisation may take 4 months or longer, and require extensive optimisation, it is relatively easy to characterise and optimise oligonucleotide primers which have

considerably less chemical complexity and, therefore, inherently more predictable properties. Moreover, while cross-reactivity is a frequent problem when selecting antibodies for protein detection, it is a simple matter to select PCR primers that are specific to a single member of a gene family, or even a particular splice variant of that gene.

We have successfully applied *in situ* RT-PCR to 1 mm paraffin-embedded tissue section arrays in order to determine which cells within a cancer are responsible for gene over-expression observed in RNA extracts. A number of technical manipulations were incorporated into the in situ protocol to ensure specificity and fidelity, and these transferred readily to the micro-array format. To our knowledge, this is the first time this procedure has been applied simultaneously to multiple samples in a microarray format.

A DNAse digestion step is commonly used in RT-PCR amplification in order to reduce the risk of spurious amplification of genomic DNA [8–10]. This can also be carried out on tissue sections [6] but the extensive incubation time required (up to 16 hr) means that considerable tissue autolysis occurs, damaging tissue structure and making post-PCR identification of cells difficult. In our protocol, the DNAse digestion step was omitted so as to better preserve tissue structure. Modifications to experimental design were employed to prevent amplification of genomic DNA. Although other approaches have been taken to obviate nuclease pre-treatment [11], we employed more conventional means. Firstly, primers were designed to amplify across two different exons, so that the amplified fragment from reverse transcribed, fully spliced mRNA would be small (300 bp), whilst the distance between the same primer sites in genomic DNA was over 3500 bp. Secondly, the number of PCR cycles and the duration of the polymerisation step were minimised so that any priming from genomic DNA would fail to achieve chain-reaction amplification. These strategies had a number of other beneficial effects: the PCR cycle number was kept with the linear range of amplification established by real-time quantitative RT-PCR, giving a more quantitative representation of the mRNA remaining in each cell, and avoiding significant synthesis of non-specific artifacts. Diffusion of reaction products away from the site of synthesis, another problem associated with *in situ* PCR [12], was reduced by this rapid procedure and exposure of the tissue sections to destructive conditions was also minimised, with the result that post-amplification staining revealed a high degree of preservation of tissue architecture and cellular features.

A consequence of using low PCR cycle numbers is that the degree of amplification will be limited, with implications for detection of the PCR product. Standard peroxidase-linked antibody detection is insufficiently sensitive.

Chemiluminescent or fluorescent detection reagents could be used instead to amplify the signal, but these would require specialised image detection systems and would rapidly diffuse away from the point of detection. Immunogold labelling followed by silver nucleation produced solid particles visible by light microscopy at magnifications suitable for visualising tissue and cellular features. This enabled simultaneous imaging of PCR products and hematoxylin-stained tissue details. The silver particles were bound to PCR products via anti-digoxygenin antibodies, and proved resistant to diffusion, remaining in the same cellular localisation as the original mRNA.

A persistent problem with the *in situ* PCR procedure has been inconsistency of results. Dedicated instrumentation has been designed with the aim of controlling conditions on a microscope slide, and some machines accommodate four or more slides to increase throughput and lower experimental variability. However, variation in the quality of paraffin-embedded tissue sections, and the number of steps involved in *in situ* PCR and the time taken to acquire data on significant numbers of samples affect the reproducibility of the technique. We found that a single, standard *in situ* PCR coverslip covered up to seventy 1 mm sections on a Clinomics tissue microarray, enabling simultaneous amplification of reverse transcribed RNA in each section under selected conditions. Although small tissue sections are more likely to become dislodged during the process of de-waxing and amplification, the use of poly-L-lysine coated slides decreased these losses and the cancer tissues examined were intrinsically more adherent due to their high cellularity. Thus a significant number of tissue samples could be analysed per single experiment. This approach substantially addresses the problem of slide-to-slide variability by subjecting large numbers of samples to identical experimental conditions. In addition, our technical modifications minimised tissue damage during preparation and amplification, preserving useful information on cellular morphology.

Conclusions
In situ PCR can be successfully applied to tissue microarrays for the specific detection and cellular localisation of transcriptional expression. The analysis of up to 70 sections in a single amplification experiment addresses the problem of experimental variability associated with this method. Modifications were introduced into the procedure aimed at reducing sample degradation, resulting in preservation of tissue and subcellular structures and reducing diffusion of labelled PCR products. Methods were carried out using entirely commercially available reagents, in order to develop a standardised procedure for visualisation of mRNA expression *in situ*.

Methods

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Real-time quantitative RT-PCR analysis of gene expression [13,14] was carried out on first-strand cDNA derived from RNA isolated from samples of normal and tumour tissues. Each PCR reaction contained 10 ng first-strand cDNA (prepared from each mRNA sample using SuperscriptTM reverse transcriptase, Life Technologies, Carlsbad, CA), SYBR green sequence detection reagents (Applied Biosystems, Foster City, CA), and sense and anti-sense primers. The primers used to amplify spondin-2 were: sense, 5'-CTCGTTTGTGGTGCGCATCGTG-3'; antisense, 5'-CAGGGAGACCTCGCAGTCCAGC-3'. The thermal cycling parameters were; 1 cycle of 94°C for 2.5 minutes followed by 40 cycles of 94°C for 40 seconds, 60°C for 50 seconds, 72°C for 30 seconds. Real-time quantitative RT-PCR was assayed on an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) and the accumulation of PCR product was measured in real time as the increase in SYBR green fluorescence. Data was analyzed using the Sequence Detector program v1.6.3 (Applied Biosystems, Foster City, CA). Standard curves relating initial template copy number to fluorescence and amplification cycle were generated using the amplified PCR product as a template, and were used to calculate mRNA copy number in each sample. Data were expressed as relative mRNA expression.

In Situ RT-PCR

Direct *in situ* RT-PCR detection of spondin-2 mRNA expression was examined in formalin fixed, paraffin embedded prostate cancer tissues (Clinomics Biosciences, Inc., Frederick, MD), arranged on microscope slides in arrays of up to 250 sections, each 1 mm in diameter. The tissue was de-waxed in xylene, gradually re-hydrated through alcohol and washed in phosphate buffered saline (PBS) before being permeabilised in 0.01% Triton X-100 for 3 minutes followed by treatment with Proteinase K for 30 minutes at 37°C. Direct *in situ* RT-PCR was carried out in a GeneAmp In Situ PCR System 1000 (Perkin Elmer Biosystems, Foster City, CA) using a GeneAmp Thermostable rTth RT-PCR kit (Perkin Elmer Biosystems, Foster City, CA). In addition to the spondin-2 specific primers described above, the following 'scrambled' primers were used for control amplifications: 5'-GTTGCGATCGTGCTGTGCGTCT-3'; 5'-CGACGCTAGCTCAGCACGCGAG-3'. The thermal cycling parameters were; 1 cycle of 94°C for 2.5 minutes followed by 20 cycles of 94°C for 40 seconds, 60°C for 50 seconds, 72°C for 30 seconds. Amplified product was detectable through the direct incorporation of alkali stable digoxigenin-11-deoxyuridine triphosphate (dUTP; Roche Diagnostics Ltd., Basel, Switzerland) which was added to the reaction mix according to the manufacturer's recommendation. After washing in PBS, 10 ul Anti-Digoxigenin-Gold antibody (Roche Diagnostics Ltd., Basel, Switzerland), diluted 1:30 in PBS and bovine serum albumin (BSA, Sigma, Dorset, UK) (1 mg BSA/1 ml PBS) was incubated on the tissue section for 30 minutes at room temperature, washed once in PBS then five times in deionised water to remove all traces of ions. 100 ul freshly prepared silver enhancement reagents (Roche Diagnostics Ltd., Basel, Switzerland) were applied to the immunogold-labelled slide and incubated for thirty minutes. The tissue was counter-stained with hematoxylin (Dako Ltd., Glostrup, Denmark) and images were captured by a digital camera attached to a light microscope.

References

1. Staecker H, Cammer M, Rubinstein R and Van de Water TR **A procedure for RT-PCR amplification of mRNAs on histological specimens** *Biotechniques* 1994, **16**:76-80
2. Steel JH, Morgan DE and Poulsom R **Advantages of in situ hybridisation over direct or indirect in situ reverse transcriptase-polymerase chain reaction for localisation of galanin mRNA expression in rat small intestine and pituitary** *Histochem J* 2001, **33**:201-211
3. Nuovo GJ **Co-labeling using in situ PCR: a review** *J Histochem Cytochem* 2001, **49**:1329-39
4. Manda R, Kohno T, Matsuno Y, Takenoshita S, Kuwano H and Yokota J **Identification of genes (SPON2 and C20orf2) differentially expressed between cancerous and noncancerous lung cells by mRNA differential display** *Genomics* 1999, **61**:5-14
5. **Ensembl** [http://www.ensembl.org/Homo_sapiens/exonview?transcript=ENST00000290902&db=core]
6. Komminoth P and Long AA **In-situ polymerase chain reaction. An overview of methods, applications and limitations of a new molecular technique** *Virchows Arch B Cell Pathol Incl Mol Pathol* 1993, **64**:67-73
7. Osada T, Uehara H, Kim H and Ikai A **mRNA analysis of single living cells** *J Nanobiotechnology* 2003, **1**:2
8. Grillo M and Margolis FL **Use of reverse transcriptase polymerase chain reaction to monitor expression of intronless genes** *Biotechniques* 1990, **262, 264**:266-268
9. Lau GK, Fang JW, Wu PC, Davis GL and Lau JY **Detection of hepatitis C virus genome in formalin-fixed paraffin-embedded liver tissue by in situ reverse transcription polymerase chain reaction** *J Med Virol* 1994, **44**:406-409
10. Martinez A, Miller MJ, Quinn K, Unsworth EJ, Ebina M and Cuttitta F **Non-radioactive localization of nucleic acids by direct in situ PCR and in situ RT-PCR in paraffin-embedded sections** *J Histochem Cytochem* 1995, **43**:739-747
11. Menschikowski M, Vogel M, Eckey R, Dinnebier G and Jaross W **In situ reverse transcriptase-nested polymerase chain reaction to identify intracellular nucleic acids without the necessity of DNAse pretreatment and hybridisation** *Anal Cell Pathol* 2001, **22**:151-158
12. Teo IA and Shaunak S **Polymerase chain reaction in situ: an appraisal of an emerging technique** *Histochem J* 1995, **27**:647-59
13. Heid CA, Stevens J, Livak KJ and Williams PM **Real time quantitative PCR** *Genome Res* 1996, **6**:986-994
14. Morrison TB, Weis JJ and Wittwer CT **Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification** *Biotechniques* 1998, **24**:954-958