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MULTIPROBE RNASE PROTECTION ASSAY WITH INTERNALLY LABELED RADIOACTIVE PROBES, GENERATED BY RT-PCR AND NESTED PCR

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1. ABSTRACT

RNase Protection Assay (RPAs) is a highly sensitive and reproducible method of quantitating the levels of specific mRNA transcripts. The introduction of the commercially available Multiprobe RPAs allow comparing and quantifying the expression of up to different mRNA species in a single sample of 1-20 μg of total RNA. To generate probes which are not commercially available, we prepared highly specific probes by RT-PCR and nested PCR. Then, after ligation of a T7 promoter, another PCR was performed with a primer set consisting of a specific sense primer and antisense T7 primer. Only the antisense strand of the double stranded PCR-product contained the T7-promoter sequence on its 5' end, allowing *in vitro* transcription and internal labeling with [α–^{32}]UTP. Probe concentration was determined in a scintillation counter and equal counts were introduced in the assay. *In vitro* transcription of the PCR generated probes resulted in radioactive probes with a very high specific activity, allowing simultaneous analysis of 70 different RNA samples. RPA could be performed under the same conditions as recommended for the commercially available probe sets, avoiding time consuming optimization of reaction conditions. Negative controls consisted of yeast RNA and sense RNA probes. Positive controls were single stranded templates, generated by asymmetric PCR. Dilution series revealed a high reproducibility and the potential of this technique to semi-quantitate mRNA in different RNA samples. In conclusion, probes may be generated by RT-PCR and nested PCR that will work with the commercially available Multiprobe RPAs. The high probe yield allows analysis of a great number of samples using the same set of probes with a high reproducibility.

2. RESULTS AND DISCUSSION

RNase ***P***rotection ***A***ssay (RPA) offers several advantages over other techniques for the detection and quantification of specific mRNAs such as Northern or dot blotting. RPA is highly sensitive and non-specific binding of labeled probes is eliminated by the RNase digestion step (2). The introduction of the commercially available Multiprobe RPAs (Pharmingen, San Diego, USA) allows comparing and quantifying the expression of up to 10 different mRNA species in a single sample of 1-20 μg of total RNA. However, if the probes are not commercially available, a technique is required to generate the probes. As the probes have to be internally labeled with [α–^{32}]UTP, the required sequences are commonly sub-cloned into plasmids containing SP6, T3 or T7 promoters (3). We have generated probes by RT-PCR followed by nested PCR that could be *in vitro* transcribed to generate internally labeled radioactive probes. RPA can be performed with these probes under the same reaction conditions as those provided by Pharmingen, a major manufacturer of commercial RPA. This technique not only saves time but also allows using the PCR-probes with the commercially available Multiprobe-sets.

We set up a Multiprobe RPA with three different probes to analyze the expression of several mRNA species. By incorporating probes for the housekeeping genes, GAPDH and the ribosomal RNA, L32, the levels of individual mRNA species could be compared between samples. The technique was used to study the menstrual cycle dependent expression of endometrial factors, which are presumably involved in the regulation of endometrial function. We analyzed ebaf (endometrial bleeding associated factor), Osteopontin and β_3-Integrin in 30 endometrial tissue samples.

Specific DNA-sequences were generated by RT-PCR and nested PCR, using 1 μg of total endometrial RNA extracted by using acid guanidinium thiocyanate-phenol-chloroform extraction (1). 2 μl of the c-DNA reaction mixture (cDNA cycle Kit, Invitrogen, Carlsbad, CA, USA) was used for the first PCR, using the following primer pairs: (ebaf: sense 5'-GATGAAGTGGGCCAAGAACTG-3'; antisense 5'-TAGAGATCCTAGCTTGTGGCC-3'), (osteopontin: sense 5'-ACCATGAGAATTGCAGTGATTTGC-3'; antisense 5'-ACCAGTTCATCAGATTC-3') and (β_3-integrin: sense 5'-GTGCTGACGCTAACTGACC-3'; antisense 5'-CATGGTAGTGGAGGCAGAGT-3'). ebaf primers yielded a 844 base pair product, osteopontin primers yielded a 408 base pair product and β_3-integrin primers yielded a 284 base pair product A second, nested PCR was then performed to acquire highly purified probes. As mismatches can lead to failure of the RPA, the use of proof reading PWO-Polymerase (Boehringer Mannheim, Indianapolis, USA) and sequencing

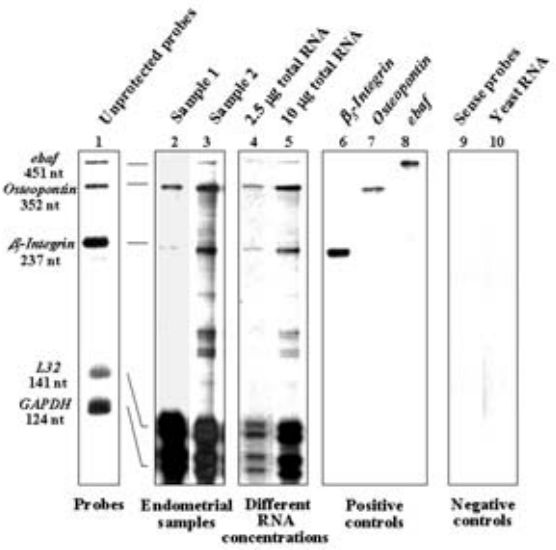

Figure 1. RNase Protection Assay (RPA) of endometrial RNA with three PCR generated radioactive probes. Probes were generated by RT-PCR followed by nested PCR. These were ligated to a T7 promoter and amplified with the specific sense primer and a T7 promoter antisense primer. Probes were *in vitro* transcribed with [α–^{32}]UTP (3000 Ci/mmol) and gel purified. RPA was performed with 5 µg of total RNA under the same conditions as recommended from the manufacturer (Pharmingen, San Diego, USA) for the housekeeping genes L32 and GAPDH. Unprotected Probes were loaded on denaturing sequencing gels as size standards (lane 1). For negative controls, we used endometrial RNA and a set of labeled RNA sense probes, instead of anti-sense probes as well as 5 µg yeast RNA instead of endometrial RNA (lanes 9-10). Positive controls were generated by asymmetric PCR from the nested PCR-products with the specific sense primers (lanes 6-8), resulting in single stranded products with the same sequence as the specific mRNA. Blank negative controls (lanes 8-9) and distinct bands in the positive controls (lanes 5-7) show the specificity of the probes. RPA with different concentrations of RNA (lanes 4-5) demonstrate low intra-assay variability and the potential of the probes for semi-quantitatation of the mRNA in different RNA samples. Analysis of endometrial tissues revealed differences in the expression of ebaf, osteopontin and β_3-integrin in tissues derived from different phases of the menstrual cycle (lanes 2-3).

of the final PCR-products were crucial. For the second round of PCR, primer pairs were chosen to create probes with different sizes, avoiding interference of the protected bands in the final electrophoretic separation. ebaf primers yielded a 451 base pair product (sense 5'-TGAACGTGTGCATAGAGGTGG-3'; antisense 5'-CATCCTTCCTCTTAGCACCCT-3'), osteopontin primers yielded a 352 base pair product (sense 5'-GTGATTTGCTTTTGCCTCCTAG-3'; antisense 5'-CATCAGACTGGTGAGAATCATC-3') and β_3-integrin primers yielded a 237 base pair product (sense 5'-CCAGGTGACCCGCTTCAATGAG-3'; antisense 5'-ACTACCAACATGACACTGCCCG-3'). All PCRs were run for 35 cycles at the same annealing temperature (52^{o}C). The PCR-products were each gel purified by resolving them in a 2% agarose gels followed by elution of the specific bands (Prep-A-Gene, BIORAD, Hercules, CA, USA). Then, a T7-promoter was added to 20 ng of the PCR-products by a ligation reaction (Lig'n Scribe, Ambion, Austin, TX, USA). This was followed by a third PCR using the specific sense primers and an antisense primer for the T7-promoter, provided by Ambion. This final PCR gave highly specific double stranded DNA-probes, enough for 20-40 *in vitro* transcriptions. As the sense strand of the double stranded PCR-product contained only the complementary T7-promoter sequence, it could not be recognized by the T7-polymerase. The antisense strand contained the T7-promoter sequence on its 5' end, allowing *in vitro* transcription and internal labeling with [α–^{32}]UTP (3000 Ci/mmol, 10mCi/ml, Amersham Life Science Inc., Illinois, USA). 30 ng of each PCR-probe and two probes for the housekeeping genes, L32 and GAPDH (Pharmingen), were separately *in vitro* transcribed (*In vitro* Transcription Kit, Pharmingen), resulting in antisense RNA-probes. To eliminate small probe fragments caused by premature termination, gel purification in 6%, 1 mm thick and 16 cm long denaturing sequencing gels was performed. Probes were cut out off the gels after visualizing by a 30 sec. exposure to X-ray films and eluted overnight in an elution buffer containing 0.5 M NH_4Oac, 20 mM $Mg(OAc)_2$, 0.1 mM EDTA, and 0.1% SDS. Probes were precipitated and dissolved in a hybridization buffer, provided with the RPA-Kit (RPA Kit, Pharmingen). Probe concentration was determined in a scintillation counter and equal counts were introduced in the assay. 3.5×10^5 cpm of the probe mixture was used for each RPA reaction. The yield of the *in vitro* transcriptions was high enough to analyze around 70 samples. RPA was performed under the same conditions as recommended for the probes provided by Pharmingen. In brief, 5 µg of total RNA was dried in a vacuum evaporator centrifuge and diluted in hybridization buffer. RNA samples were mixed with the probe sets and hybridized for 14-16 hours at 56°C. To reduce assay variability, 30 samples were analyzed at once in one experiment. Non-hybridized RNA and free probes were digested by incubation with RNase A and T1 for 45 min. at 30°C. Enzyme activity was stopped by the addition of Proteinase K. The non-digested, RNase protected, RNAs were purified by phenol/chloroform extraction and resolved on a denaturing 6% acrylamide sequencing gel for 2 hours at 50 Watt. Unprotected probes were loaded as size markers. Gels were dried for 1 hour at 80°C degrees and exposed to X-ray films with an intensifying screen for 3-20 hours. Identity of the RNase protected bands was established by comparing the migration distance of the bands with those of the unprotected probes and by adding positive controls. Positive controls were generated by asymmetric PCR of the nested PCR-products: 40 cycles were run only with the antisense primer, resulting in single stranded products with the same sequence as the specific mRNA. For negative controls, we analyzed 5 µg yeast RNA instead of endometrial RNA. Another negative control consisted of a set of labeled sense RNA probes - instead of anti-sense probes - which were hybridized to 5µg of total endometrial RNA. Semi-quantification was achieved by normalizing the

optical densities of the specific bands to the optical densities of the housekeeping genes, L32 and GAPDH. The optical density of the specific bands was expressed as relative values.

In vitro transcription of the PCR generated probes resulted in a high probe concentration, enough to analyze 70 RNA samples (data not shown). Each probe was tested separately in an RPA to exclude the appearance of non-specific bands of a size that could interfere with other protected probes (figure 1, lane 6-8). To identify the RNase protected bands, we loaded unprotected probes as size markers on each gel (figure 1, lane 1). The unprotected probes for ebaf, osteopontin and β_3-integrin had the same size as the protected bands. The unprotected probes of the housekeeping genes, L32 and GAPDH (Pharmingen), were 28 nucleotides longer than the protected bands due to flanking sequences that did not hybridize to the target RNAs. Negative controls showed no bands at all, excluding residues of undigested probes (figure 1, lane 9, 10). As the mRNA expression had to be semi-quantified, we calculated the relative optical densities of the protected bands in samples with different RNA-concentrations (figure 1, lane 4, 5). A 2-fold increase of the relative optical density values corresponded to a 1.5-fold increase of the specific RNA. The reproducibility of the RNase Protection Assay was determined by comparing the relative optical density values of the same samples, analyzed in the same and in different assays. The intra-and inter-assay variation of the relative optical density values was <10%.

In conclusion, probes may be generated by RT-PCR and nested PCR that will work with commercially available Multiprobe RPAs. The high probe yield allows analysis of a great number of samples with the same set of probes with a high reproducibility.

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4. REFERENCES

1. Chomczynski, P. and N. Sacchi. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156-159. (1987)

2. Gillman, M. Ribonuclease protection assay, pp 4.7.1-4.7.8. In F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Deidman, J.A. Smith and K. Stuhl (Eds.), Current Protocols in Molecular Biology. John Wiley and Sons, Inc., New York. (1993)

3. Sambrook J., E.F. Fritsch and T. Maniatis. Molecular Cloning: A Laboratory Manual, 2nd ed. CSH Laboratory Press, Cold Spring Harbor, NY. (1989)

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