2. BioSensor
SYMPOSIUM
Tübingen 2001

Detection of mRNA using the BIACORE

B. Henze, C. Bebber, J. J. van den Heuvel, U. Bilitewski

Gesellschaft für Biotechnologische Forschung mbH, Mascheroder Weg 1, D-38124 Braunschweig

ubi@gbf.de   www.gbf.de

Abstract

We present the detection of native mRNA using the BIACORE system. The influence of different probes and flow rates on the detection is shown and compared to the hybridisation of oligonucleotides. Probes for mRNA detection were chosen by calculations of secondary structures using energy minimizing criteria based on the algorithm of Zuker [1]. Probe concentrations were optimised as well as the regeneration conditions for the sensor surface. The influence of the flow rate appeared to be more marked for mRNA than for oligonucleotide hybridisation.

1 Introduction

Pharmaceutical relevant proteins like growth factors are produced by recombinant microorganisms such as *E. coli* or *Pichia pastoris*. To optimise process yields it is not only necessary to optimise cultivation conditions but also strains that offer an efficient protein induction. First step of the intracellular protein synthesis is the transcription of the gene into mRNA followed by the translation into the protein. Therefore the efficiency of protein production is to be investigated not only on the protein level but also on the mRNA level. mRNA is a single-stranded polynucleotide possessing similar specific hybridisation properties as DNA. That is why the expression of a certain gene may be monitored by hybridisation of a specific probe. In the classical Northern blot analysis these probes are labelled with radioactive $^{32}PO_4$ for the identification of a specific mRNA. New methods of mRNA analysis are based on so-called gene-chips allowing the simultaneous detection of up to several thousand genes [2]. However, the cellular mRNA has to be transferred into fluorescent cDNA (reverse transcription). Label free detection of affinity reactions is well established for protein analysis and the analysis of the hybridisation of oligonucleotides [3,4]. As this approach avoids the reverse transcription reaction, we wanted to investigate the applicability of the BIACORE to mRNA detection.

As an example the mRNA of bFGF, the basic fibroblast growth factor, was chosen. bFGF is an 18 kDa protein which was produced by recombinant *E. coli* strains [5]. mRNA from prokaryotic cells such as *E. coli* lack a polyadenylated sequence at the 3'-end that is present in eukaryotic cells and often used as recognition site for total mRNA. Prokaryotic mRNA can only be detected by sequence specific oligonucleotides. mRNAs, however, exist in multiple secondary structures of different stability which may hamper or even inhibit the access of probes to their recognition sites. In Northern blot analysis the secondary structures are broken by denaturation whereas in the BIACORE system the native

mRNAs are to be used. The choice of suitable oligonucleotides is therefore crucial. First tests, however, were carried out with mRNA obtained by *in-vitro*-synthesis, as this allows a more precise calibration of the assay as only one mRNA-product is obtained.

2 Experimental

2.1 *in-vitro*-mRNA-synthesis

The production of run-off-transcripts was done using a plasmid containing a T7-promoter that contained a gene coding for bFGF (basic fibroblast growth factor) followed by an polyA-sequence that enables the production of polyadenylated mRNA. *In-vitro*-mRNA was made using the T7-transcription-kit by Promega (Promega, Large Scale RNA Production System – T7, Cat.Nr. P1300). The purification and characterisation was done following instructions of the supplier using the kit mentioned above.

2.2. Secondary structure calculations for probe optimisation

Probes were chosen using the programs mfold (calculating) and plotfold (displaying) out of the GCG-package [a] in order to look at the secondary structure of the mRNA. Mfold calculates energy matrices that determine all optimal and suboptimal secondary structures for an mRNA molecule using energy minimization criteria [1].

Different optimal foldings are calculated when the folding energies differ only slightly. Therefore a sequence of the mRNA should be chosen showing an open loop in most calculated optimal and suboptimal secondary structures. This increases the probability for a reliable prediction of such a structural element compared to regions with higher structural variation.

Furthermore considerations about the melting temperature of the resulting double-strand and primer dimerisation were taken into account. Melting temperatures were calculated with: Tm = n(A,T) x 2 °C + n (G,C x 4°C) [6]. They should be significantly higher than room temperature to lead to a stable binding. Primer dimerisation is to be avoided. The following oligonucleotides were chosen, of which Hyb1, Hyb2, polyT and PCR2 were labelled with biotin at the 5'-end:

```
HYB1:   324-340 bps; 17-mer; TGT AGT TGT TGG ATT CT          Tm: 46°C

HYB2:   457-473 bps; 17-mer; TAT TAG GAT TTA GCG GA          Tm: 46°C

polyT:               24-mer; TTT TTT TTT TTT TTT TTT TTT TTT  Tm: 48°C

PCR2:   5-29 bps;    24-mer: TGG CAG CAG GAT CAA TAA CAA CAT  Tm: 68°C

full-m:              24-mer; ATG TTG TTA TTG ATC CTG CTG CCA  Tm: 68°C

20-m:                20-mer; ATG TTG TTA TTG ATC CTG CT       Tm: 54°C

18-m:                18-mer; ATG TTG TTA TTG ATC CTG          Tm: 48°C

16-m:                16-mer; ATG TTG TTA TTG ATC C            Tm: 42°C

14-m:                14-mer; ATG TTG TTA TTG AT               Tm: 34°C

12-m:                12-mer; ATG TTG TTA TTG                  Tm: 30°C

10-m:                10-mer; ATG TTG TTA T                    Tm: 24°C
```

Sequence of bFGF (478 bps) [7], with minor modifications:

```
1     CATATGGCAG   CAGGATCAAT   AACAACATTA   CCAGCTCTGC
41    CGGAAGACGG   TGGTTCTGGT   GCTTTCCCCC   CGGGTCACTT
81    CAAAGACCCG   AAACGTCTGT   ACTGCAAAAA   CGGTGGTTTC
121   TTCCTGCGTA   TCCACCCGGA   CGGTCGTGTT   GACGGTGTTC
161   GTGAAAAATC   CGACCCGCAC   ATCAAACTCC   AACTGCAGGC
201   TGAAGAACGT   GGTGTTGTTT   CCATCAAAGG   TGTTTGCGCT
241   AACCGTTACC   TGGCTATGAA   AGAAGACGGT   CGTCTGCTGG
281   CTTCCAAATG   CGTTACCGAC   GAATGCTTCT   TCTTTGAACG
321   TCTAGAATCC   AACAACTACA   ACACCTACCG   TTCCCGTAAA
361   TACACCTCCT   GGTACGTTGC   TCTGAAACGT   ACCGGTCAGT
401   ACAAACTGGG   TTCGAAAACC   GGTCCGGGTC   AGAAAGCTAT
441   CCTGTTCCTG   CCGATGTCCG   CTAAATCCTA   ATAAGCTT
```

2.3 mRNA analysis using the BIACORE

BIACORE measurements were performed using a BIACORE 2000 (Biacore AB, Uppsala, Sweden). Sensor chips were CM5-chips (BR-1000-14, Biacore, Freiburg) with a carboxymethylated dextran-hydrogel surface on a gold layer. Streptavidin (100µg / mL resulting in approx. 10,000 RU) was covalently coupled using EDC/NHS chemistry [8]. All used buffers and solutions were filtered (0.2 µm) and degassed. As running and injection buffer 2x SSC (30 mM sodium citrate, 0.3 mM sodium chloride) was used. Regeneration was done by flushing the chip with 5 µL of 10 mM NaOH. The oligonucleotides Hyb1, Hyb2, PCR2 and polyT were attached onto the streptavidin surface by a biotin-label (600 pmol each). Solutions of the analytes (*in-vitro*-mRNA or equivalent oligonucleotides and full-m, 20-m, 18-m, 16-m, 14-m, 12-m, 10-m) were injected using flow rates from 1 to 10 µL / min (for oligonucleotides 600 pmol each). All assays were performed at 25°C.

2.4 Optimisation of the probe concentration

To evaluate the optimal probe concentration different biotinylated probe concentrations were tested (0.01µmol / L to 10 µmol / L).

For each experiment a fresh streptavidin flowcell was used. The probes were dissolved in 2x SSC and flow rates of 5 µL / min were employed.

2.5 Optimisation of the regeneration step

Different concentrations (2.5 mM and 5 mM) of HCl instead of NaOH as regeneration reagent were tested.

3 Results and Discussion

3.1 Single-stranded oligonucleotide detection

The use of oligonucleotide PCR2 as a probe allows the detection of the oligonucleotides representing full matches (full-m) down to just 10 matches (10-m). The corresponding signal was measured in two different flow channels.
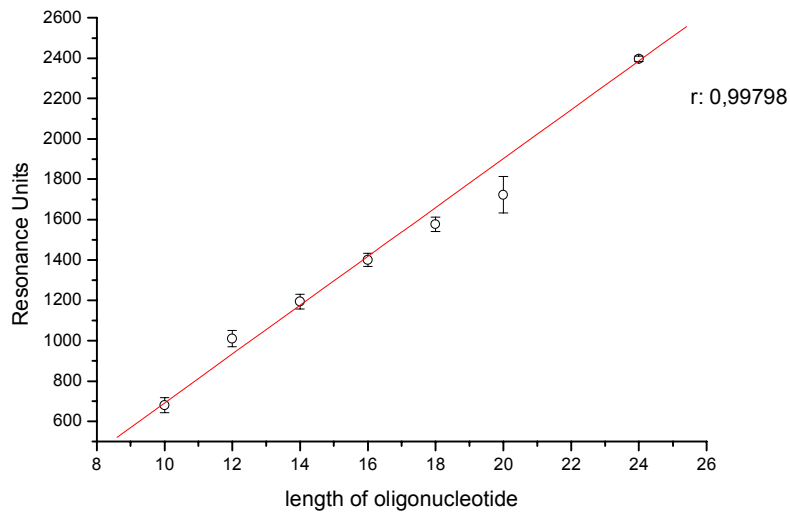
Fig. 1: Hybridisation of oligonucleotides of different length
(each point represents the mean value of two different flow-channels, flow rate: 5 µL / min)

Fig. 1 shows a good linear dependency between the length of the sample oligonucleotide and the resulting signal on the sensor surface. The variation between the two different flow channels is indicated by the error bars (~ 5%).

This correlates with other investigations showing that the affinity increases with number of matching basepairs and with the melting temperature Tm, in particular a linear relationship between affinities and calculated Tm using 7-mer to 10-mer oligonucleotides towards the same probe was shown [4]. Here we showed the linearity over a wider range of matches towards the same probe.
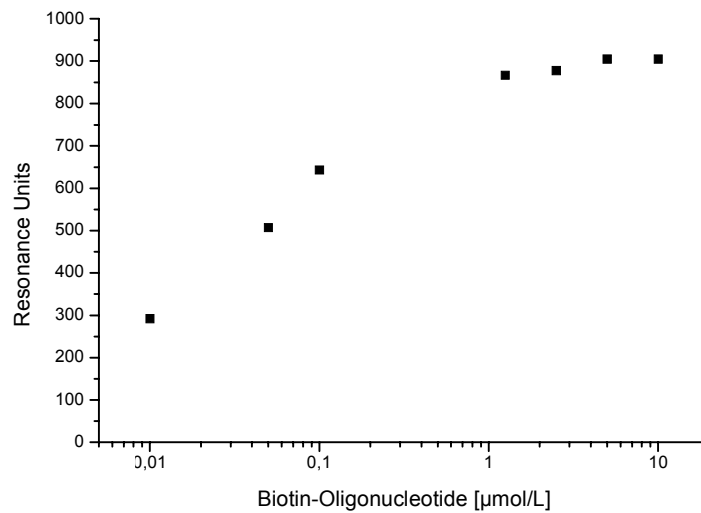
3.2 Optimised probe concentration

Fig. 2: Optimisation of biotinylated probe concentration

Fig. 2 shows that the signal increases with probe concentration and saturation is reached at about 2 µmol / L. In the following much higher concentrations of biotinylated probe were used to compensate the variation in streptavidin on the sensor surface.
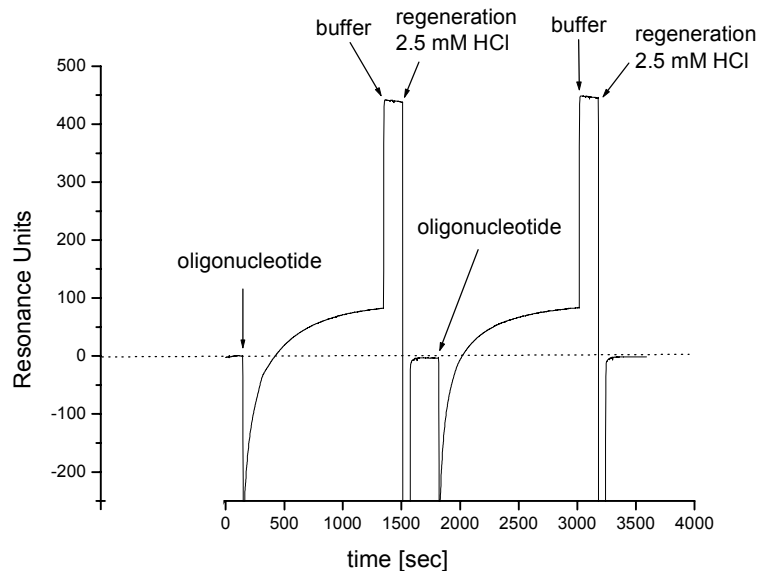
3.3 Regeneration of the probe surface

Fig. 3: Regeneration of probe surface using HCl

The regeneration of the sensor surface for oligonucleotides is usually done by 1 min. flushes of 50 mM NaOH. As observed by Kuhlmeier [b] this causes significant loss in the hybridisation signal already after several regenerations (34% after 20 injections). Therefore we tested the applicability of HCl in

different concentrations. We noticed that concentrations of only 2.5 mM HCl were sufficient in most cases for complete regeneration of the sensor surface without notable loss of signal (Fig. 3). 5 mM HCl showed a 10 times stronger baseline drift than 2.5 mM HCl.

3.4 Influence of the flow rate
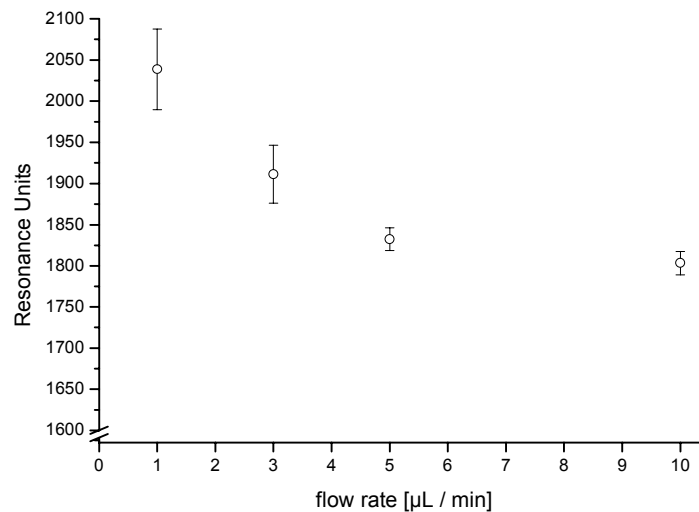
Fig. 4: Flow rate dependency of 24-mer hybridisation (full-m)
(each datapoint represents the mean value of three hybridisations)

Decreasing the flow rate leads to extended contact times between the probe and its corresponding oligonucleotide. On the other hand the mass transfer to the sensor surface is slower due to the more extended diffusion layer. Thus, the influence of the flow rate on resulting hybridisation signals is to be investigated. In Fig. 4 the results are summarized for the full-match oligonucleotide. A continuous decrease with increasing flow rate was observed up to flow rates of approx. 5 µL / min.

Thus, the reduced contact time seems to be more important than the accelerated mass transfer, i.e. the hybridisation reaction was the rate limiting step.

3.5 mRNA detection
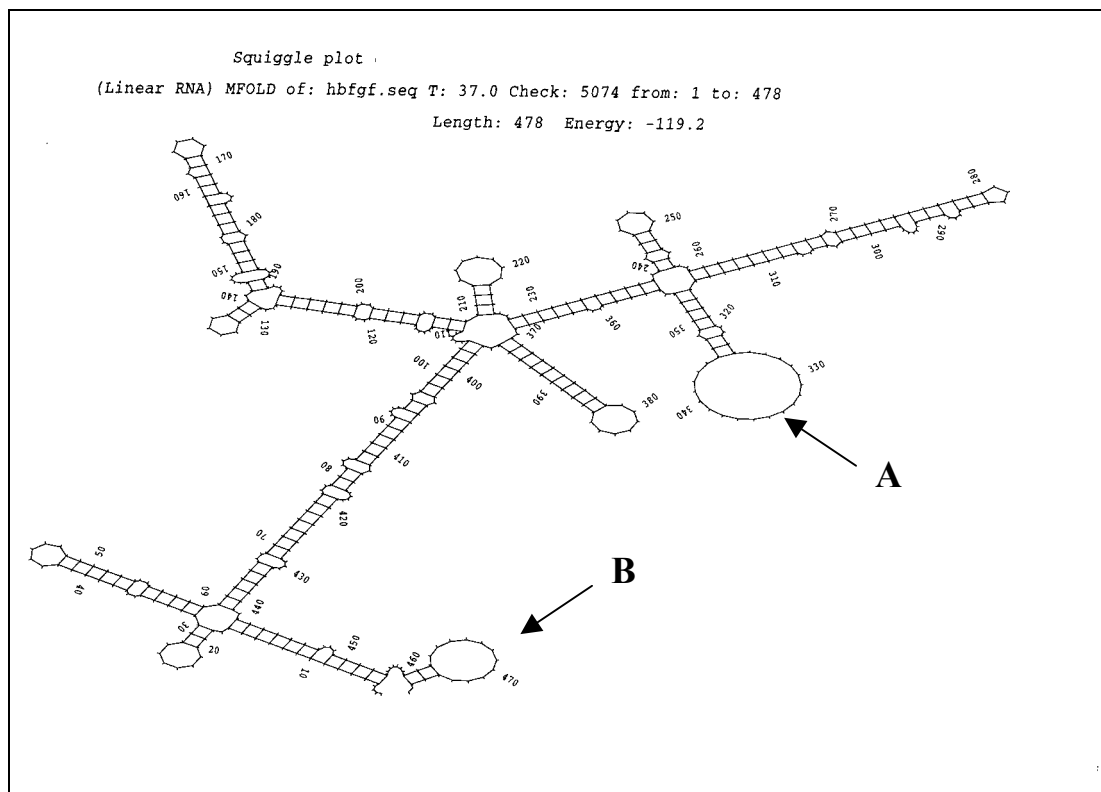
Fig. 5: Calculated mRNA secondary structure using Zuker algorithm,
A: position of probe Hyb1, B: position of probe Hyb2

Detection of mRNA without any modifications should be possible using direct affinity sensors, such as the BIACORE system. Different oligonucleotides were chosen according to calculated structures of the mRNA to be analysed (Fig. 5 as an example). These calculations were performed using the programs mfold and plotfold from the GCG-software package [a] using the Zuker-algorithm [1]. Usually for a given sequence of nucleotides different structures are suggested differing in the resulting free energies. Thus, no defined constant picture of the nucleic acid can be given. It has to be assumed that all calculated structures are present in a dynamic equilibrium, so that some substructures are conserved whereas others show high dynamics. Well-determined substructures are predicted correctly with a higher probability than poorly determined structures [c]. For the selection of sequences suitable for probe hybridisation regions were chosen which showed open loops in a relatively high number of those calculated structures, thus seemed to be conserved loops. Positions of the used probes are mentioned in 2.2 and are shown in Fig. 5 (A: hybridisation site for Hyb1, B: hybridisation site for Hyb2). As hybridisations to Hyb1 and Hyb2 were successful (see following figures), it can be assumed that these sites in the mRNA represent open loops with a high probability.
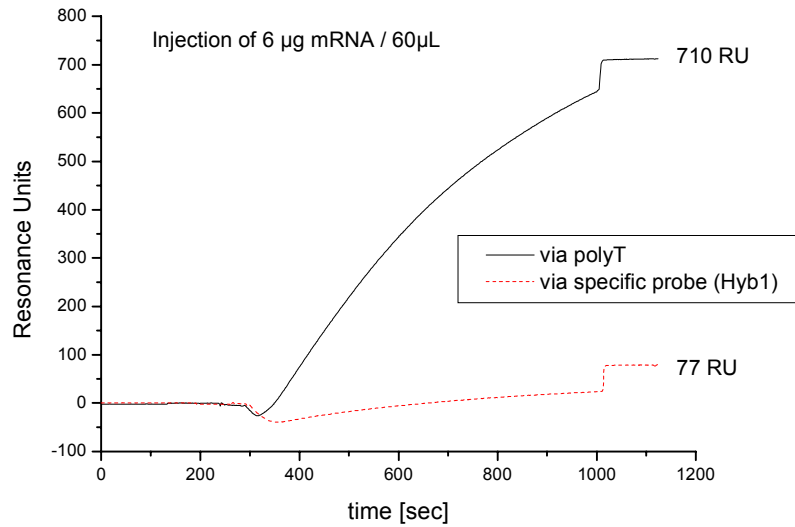
Fig. 6: Hybridisation of mRNA via a polyT and via a specific probe

The strongest hybridisation signals were obtained using the polyT-probe that recognises the polyadenylated sequence of the *in-vitro*-mRNA because numerous binding possibilities are present (Fig. 6). The gene specific probes (Hyb1 and Hyb2) showed a hybridisation signal 10 times weaker than the polyT-probe with the Hyb2-signal being 5 times lower than the Hyb1-signal (data not shown).
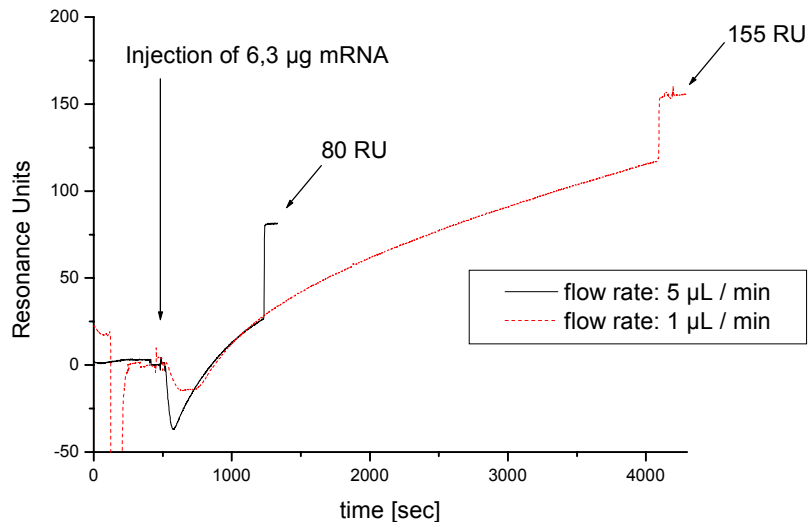

Fig. 7: Influence of the flow rate to the hybridisation of mRNA using Hyb1 as probe

The influence of the flow rate was investigated as well (Fig. 7). In order to achieve higher sensitivity the flow rate was switched from 5 to 1 µL / min. This almost doubled the signal. A lower flow rate favours binding events that need more time to take place. The association is favoured because the

mRNA has got more time to access their binding sites by twisting and bending. Higher flow rates reduce the probability of the right position at the right time. Again, the hybridisation is the rate limiting step.
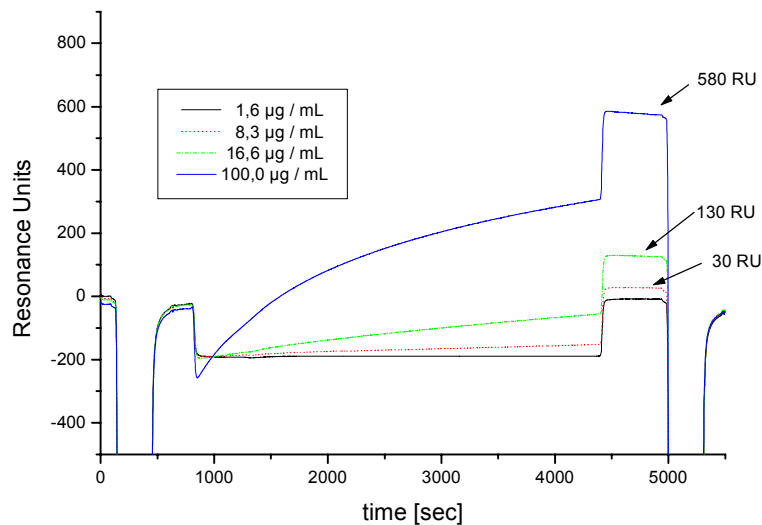

Fig. 8: Lower detection limit of bFGF-mRNA using Hyb1 as probe

The lower detection limit for bFGF-mRNA (478 bps) using the BIACORE and Hyb1 as probe was approx. 15 µg / mL with a flow rate of 1 µL / min (Fig. 8).

Trying to denature the mRNA prior to injection was unsuccessful. The hybridisation showed no difference or even lower signals, probably because of the fast refolding of the secondary structure or even hydrolysis of mRNA.

4 Conclusion

First tests have shown that mRNA can be detected in the BIACORE instrument in native state without the need for any further pre-treatment. Investigations on a suitable probe length showed that signals increase with increasing numbers of matching base pairs. Thus, the chosen probe with a length of 17 bp should give reliable signals, which, however could eventually increased to some degree by a longer probe. The influence of the flow rate seemed to be more marked for mRNA detection than for oligonucleotide hybridisation. Obviously, the hybridisation event is the rate limiting step in both cases. Reducing the flow rate from 5 to 1 µL / min lead to a two times higher hybridisation signal for the mRNA, whereas the oligonucleotides showed only a 10% increase. The difference in the hybridisation from mRNA towards the probes Hyb1 and Hyb2 shows again that the hybridisation is the rate limiting step and is probably due to different accessibilities of the corresponding regions on the mRNA. Absolute values obtained with the BIACORE for the amount of bound analyte are comparable as long as the different flow channels on a common sensor chip within a sequence of experiments are used. However, variations of absolute values are observed, when different chips are compared, or when the chip is stored for an extended period of time. This does not influence the principal results. Thus, the calibration curve to be used for quantitative determinations of mRNA is to be obtained from the same sensor chip used for the analysis.

5 Outlook

As observed by Schuderer et al. [9] fluorescence-labelling and the use of a total internal reflection fluorescence (TIRF) device can improve lower detection limits significantly compared to the BIACORE. This requires, however, the introduction of fluorophores into the analyte. This can occur either by transcription of the mRNA into cDNA using fluorescently labelled nucleotides or by using a second, fluorescently labelled probe. Due to the extent of additional investigations corresponding results will be published separately.

6 References

[1]   Zuker, M. (1989) *Science*, **244**, 48.

[2]   Lipshutz, R.J., Fodor, S.P.A., Gingeras, T.R., Lockhart, D.J. (1999) *Nature genetics suppl.*, **21**, 20.

[3]   Rich, R.L., Myszka, D.G. (2000) *J. Mol. Recog.*, **13**, 388.

[4]   Persson, B., Stenhag, K., Lisson, B., Larsson, A., Uhlen, M., Nygren, P.-A. (1997) *Anal. Biochem.*, **246**, 34.

[5]   Seeger, A., Schneppe, B., McCarthy, J.E.G., Deckwer, W.-D., Rinas, U. (1995*) Enyzme Microb. Technol*., **17**, 947.

[6]   Suggs et al. (1981) *ICN-UCLA Symp. Dev. Biol.*, **23**, 683.

[7]   Knoerzer, W., Binder, H.-P., Schneider, K., Gruss, P., McCarthy, J.E.G., Risau, W. (1989) *Gene*, **75**, 21.

[8]   Johnsson, B., Löfas, S., Lindquist, G. (1991) *Anal. Biochem.*, **198***,* 268.

[9]   Schuderer, J., Akkoyun, A., Brandenburg, A., Wagner, E. (2000) *Anal. Chem.*, **17**, 3942.

[a]   http://www.accelrys.com/products/gcg_wisconsin_package/

[b]   http://opus.tu-bs.de/opus/volltexte/2000/95

[c]   http://bioinfo.math.rpi.edu/~zukerm/articles/s-annotate/