Universität Kassel

- Fachbereich für Mathematik und Naturwissenschaften, Institut für Biologie -

DNA-based Detection of Proteins

Diplomarbeit zur Erlangung des akademischen Grades Diplom Biologe

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Abbreviations

EtOH	Ethanol
Exo7	Exonuclease VII
Fil-A	Filamin A
FT	flow through
min	Minute
O	Oligonucleotide
o/n	over night
oW	flow through for separating DTT from primer
P	Primer
PAA	Polyacrylamide
PB	pink buffer
RCA	rolling circle amplification
RCP	rolling circle product
SP	signaling pathways
Sulfo-SMCC	sulfosuccinimidyl 4-[*N*-maleimidomethyl]cyclo hexane-1-carboxylate
sW	flow through for separating Sulfo-SMCC from protein
TEM	transmission electron microscopy
T_M	Melting temperature
W	flow through for protein buffer exchange to p-buffer
WB	western blot
SH	thiol group

Content

1 Introduction

Detection of molecules in biological samples is a challenging field in public healthcare. Immunoassays are the most promising methods for this task. The generation of specific antibodies and the subsequent verification are the two major tasks for immunoassays. The theory and application for immunoassays was conceived in the 1950s (Unger RH 1959). The usefulness of immunoassays derives from the biochemical characteristics of antibodies. Antibodies are able to distinguish molecular structural entities and are easy to produce, especially with the invention of hybridoma technique for the production of monoclonal antibodies (Kohler and Milstein 1975). The high binding affinities of antibodies are a beneficial character for immunoassays (Ekins 1989). Scientists discussed the question of the comparative or analytical nature of immunoassays. Signals derived in immunoassays are based on the detection of labeled antibodies. An immunoassay measures one molecular structure recognized by one antibody. This is an analytical method of measurement. Failures in comparable results are based on the samples, not on the assay itself (Ekins 1989). Measurement of a high excess (1000 times or higher) of analyte inside a medium with antibodies results always in the same fraction of bound antibody. The detection of this fraction is the principle of the ELISA. To narrow down the area of the measurement is the idea of microarrays, made by Ekins in 1989 (Ekins 1989). He made some hypothetical assumptions, which are the basis for the further development of immunoassays as an analytical instrument. The first statement of Ekins is about free mass transport. The immunoassay or better the interaction of ligand and binder should not be limited by steric hindrances or mass transport. The second statement deals with the heterogeneity of the assay. The microspot assay, for what this project of the RCA linked immunoassay aims at, has a much higher concentration of the ligand as ligand–binder complex (Kusnezow, Syagailo et al. 2006). Without mixing in the area above the binder a concentration gradient is build. The more afar the fluid (and therefore the ligand) is from the immobilized interaction partner, the less likely is the interaction of analyte and binder. Only Brownian motion moves the molecules. A dilution of antibody, due to the binding to immobilized interaction partner on the surface results in heterogeneity of the antibody in the fluid. A thermodynamic equilibrium is not present at this time. Kusnezow *et al*. (Kusnezow, Syagailo et al. 2006) showed the beneficial effect of stirring the fluid to compensate

for the heterogeneity. In a classical ELISA assay or on protein microarrays the immobilized partners are detected by one specific antibody or after incubation with a secondary antibody (Sandwich assay). Signals gained from antibodies are derived by labels, which can be either direct or indirect. Direct labels are on the antibody itself, like fluorescent dyes (AlexaFluor (Panchuk–Voloshina, Haugland et al. 1999), Cyanin-dyes (Blower, Feric et al. 2007) or fluorescein). A common disadvantage of direct labeled AB's is the restricted field of application for the antibody. The detection of the target and signal generation is done by two independent reactions / AB's. The advantage is less background and a higher sensitivity. Only if the first and the second antibody recognize the respective target a signal is generated. The target of the first AB is often the Fc part of the secondary AB. The labeled secondary AB can now be used for signal generation for different targets by recognizing another unlabeled highly specific AB. This method is called indirect detection of immunoassays. Another indirect method are enzymes linked to AB's, like alcalic phosphatase or horseradish peroxidase (Straus 1968), yielding visible signals. The use of indirect signal generation holds the advantage of signal amplification, but always needs a second reaction. Further developments of antibody labels is the immobilization of gold on the AB (Zhang, Li et al. 2010). Detection of bound AB is performed with transmission electron microscopy (TEM) as a direct detection method. Gold can also be used as a label for detection in electrochemical biosensing (Tang, Su et al. 2010). This method uses a combination of irregular shaped gold nanoparticles for labeling the AB, which are immobilized while detection of target, resulting in a higher conductivity in the current conducting area. An increase of voltage is the signal of bound and gold labeled AB in electrochemical assays. For combination of the advantage of the signal localized to the bound AB of the direct measurement and the signal amplification is the labeling of AB's done with DNA. Labeling an AB with DNA can be done on the target recognizing AB itself (direct detection) or one second antibody (indirect detection).

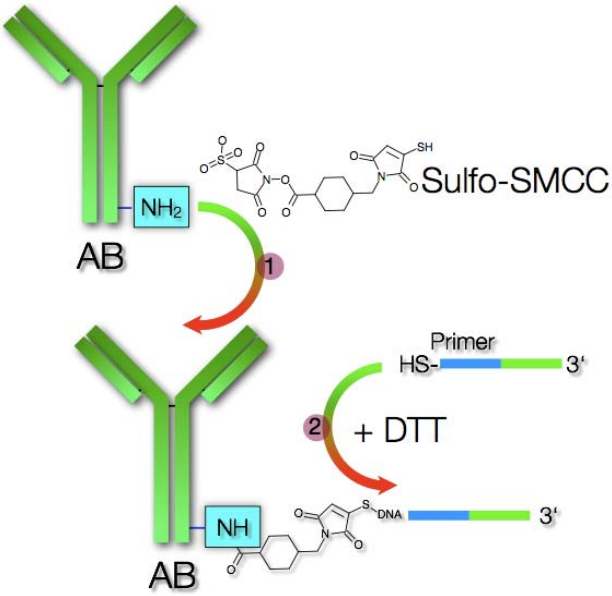


Figure 1: Two step conjugation of DNA to AB, using a thiol modified primer and the free amins of the antibody with Sulfo-SMCC as crosslinker.

The general principle for conjugation of DNA-primer to the AB is outlined in Figure 1. Conjugation is done with the heterobispecific crosslinker Sulfo-SMCC, which reacts with the free primary amines of the AB (Figure 1, step 1). The primer consists of a hybridization site for the circular template DNA (green) and a spacer sequence (blue) (Schweitzer, Roberts et al. 2002) with a thiol group at the 5' end. The reduced thiol group builds a disulfide bond with the NHS-ester of the Sulfo-SMCC (Figure 1 step 2). The DNA label holds the advantage of signal amplification, using common techniques for DNA amplification, like the polymerase chain reaction (PCR). The PCR require special heating and cooling steps and appropriate devices that tolerate high temperature differences. High temperatures results in loss of target–AB interaction and a delocalization of signal and target (Niemeyer, Adler et al. 2007). The principle of rolling circle amplification (RCA) is an isothermal method for DNA amplification (Fire and Xu 1995), resulting in an amplified DNA still bound to the target. The RCA takes advantage of the strand displacement activity of DNA polymerases derived from phages. Base pairing of a primer to a circular single stranded DNA (Figure 2 A) is a prerequisite and serves as starting point for the isothermal (Figure 2 B). The resulting RCA-product (RCP, light orange) contains multiple concatemeric copies of the template DNA (Figure 2 C).

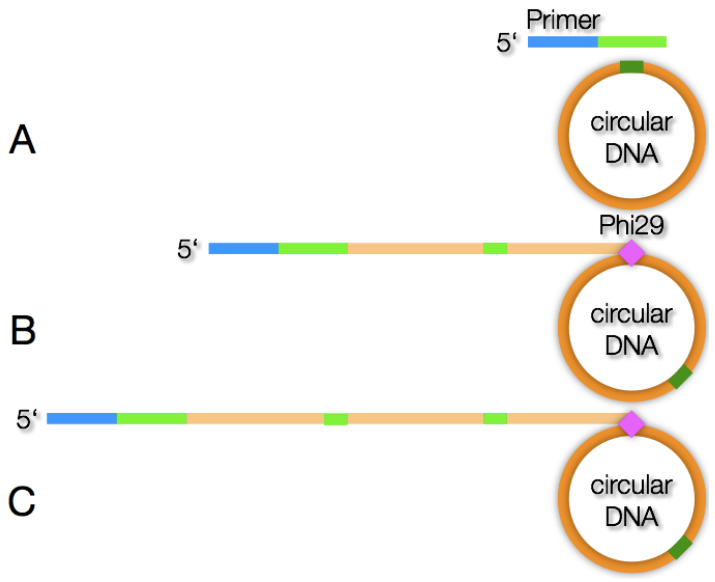


Figure 2: Principle of the RCA. A: Hybridizing linear primer with circular template DNA B: starting site for the Phi29-Polymerase C: amplification of the template DNA leading to concatemeric rolling circle product

The resulting rolling circle product (RCP) can be detected using different labels by incorporation of fluorescent nucleotides, or indirect ones like hybridizing labeled probes. Cheng and coworkers (Cheng, Yan et al. 2010) developed an RCA based method for hybridizing labels to the RCP, which is labeled with quantum dots. Quantum dots are nanostructures, able to emit light 20 times brighter as any known dye and is 100 times more resistant to photobleaching (Maureen A Walling 2009). In the developed method DNA amplification is done directly on the AB. Phi29-Polymerase uses the primer conjugated to the AB and a circular template DNA for generation of copies of the target DNA that is still linked to the AB (Figure 3). The detection of amplified DNA can be done as described above. Another detection method uses the attached DNA as template for a PCR to achieve signal enhancement rates far beyond the fluorescence measurements. A commercial available system is the PCR based amplification of oligonucleotides by Chimera Biotech®, called IMPERACER-System. The conjugation product is an antibody with a double stranded DNA. The dsDNA serves as template for the PCR. In an ELISA the IMPERACER technique results in a 1000 fold higher diction rate compared to competitive ELISA (Niemeyer, Adler et al. 2007).

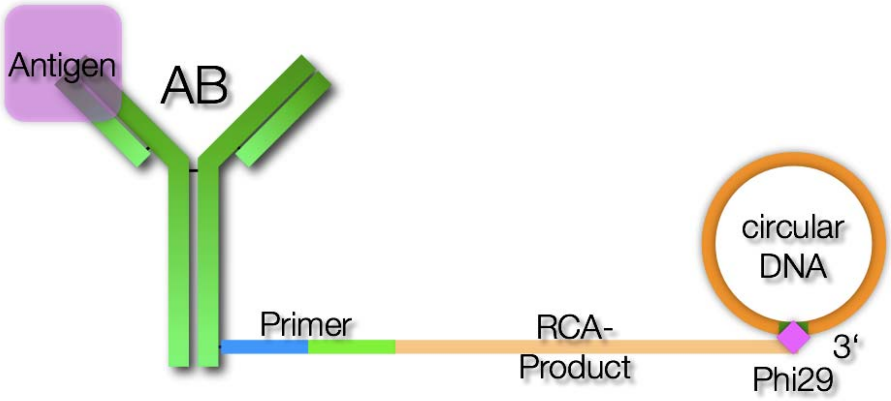


Figure 3: Scheme for the DNA-AB conjugation product and the subsequent rolling circle amplification using the Phi29 polymerase on the conjugate. The rolling circle product (RCP) is covalently bound to the AB and can be detected.

In the Diploma thesis the preparation of DNA-AB conjugate was established. The linear primer conjugated to AB and hybridized with a circular template DNA serves as starting site for the Phi29-Polymerase. Therefore the template preparation was optimized. The specificity of the antibody was tested and DNA-AB conjugate used as template for rolling circle amplification. For the detection of the modified antibody and the rolling circle product several separation and staining methods were compared. DNA detection can be done by staining gels as done by (Fire and Xu 1995) or detected in immunoassays with incubation of Cy3-labeled nucleotides, as well as with incorporation of biotin labeled nucleotides and subsequent Streptavidin-Cy3 interaction.

2 Material and Methods

2.1 Material

Antibodies

Antibody	Concentration	Species of origin	Company
αGST-AB	$5\frac{mg}{ml}$	derived from goat	GE Healthcare #27-4577-01
αGoat-IGG-Cy5	$0.2\frac{mg}{ml}$	derived from donkey	
αDonkey–Cy5	$0.2\frac{mg}{ml}$	derived from rabbit	
αGOAT-AB-Cy3	$1.5\frac{mg}{ml}$	derived from donkey	Jackson ImmunoResearch, #705-165-147PA, USA
αActin-AB	$0.2\frac{mg}{ml}$	derived from rabbit	Santa Cruz Biotechnology # sc-1616-R
αRabbit-AB–Cy5	$1.5\frac{mg}{ml}$	derived from goat	Jackson ImmunoResearch
αHis-AB	$5\frac{mg}{ml}$	derived from mouse	GE-Healthcare #27-4577-01
αMouse-AB-Cy 5	$1.5\frac{mg}{ml}$	derived from goat	Jackson ImmunoResearch

Buffers

TBS

20 mM Tris-HCl, 150 mM NaCl, pH 7.5

TBS-T

20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween20, pH 7.5

CCE-buffer

50 mMTris-HCl (pH6.8); 100 mM DTT; 2% SDS; 0.1% bromphenol blue; 10% glycerol;

Pink buffer

2.5 – 2.8 M (21.6 g) Urea; 0.8 ml Pharmalyte; 1.6 g CHAPS; 0.4 g DTT in 62.5 ml A. bidest

Buffers for Blue Native PAGE according to (Wittig, Braun et al. 2006)

	Cathode buffer B (1L)	Cathode buffer B/10	Anode buffer	Gel buffer (3x)
Tricine (mM)	50 (8.9 g)	50 (8.9 g)	-	-
Imidazole (mM)	7.5 (0.51 g)	7.5 (0.51 g)	25 (1.7 g)	75 (5.1 g)
Coomassie blue G-250 (%)	0.02	0.002	-	-
pH	7.0	7.0	7.0	

Blotting buffer

39 mM Glycin, 48 mM Tris, 0.0375 % SDS, 20 Methanol, pH 8.5

1L: 2.93 g Glycin, 5.81 g Tris, 3.75 ml 10% SDS, 200 ml Methanol, set to 1 L with A. bidest

DTT

100 mM from 1 M stock, stored at -20 °C

EDTA

Stock 0.25 M; pH 8.0

Exonuclease VII Reaction Buffer (5X)

250 mM Tris-HCl (pH 7.9); 250 mM Sodium phosphate (pH 7.8); 50 mM 2-mercaptoethanol; 42 mM EDTA.

Native loading buffer

50 mM Tris – HCl pH 7; 10 Glycerol; 100 mM DTT; 0.1 % Bromophenol Blue

Phosphate-Buffer (P-buffer)

45.83 mM Na_2HPO_4; 9.16 mM KH_2PO_4; 150 mM NaCl; 20 mM EDTA

PBS

137 mM NaCl, 2.7 mM KCl, 12 mM Phosphate

PBS with 5 mM EDTA

137 mM NaCl, 2.7 mM KCl, 12 mM Phosphate, 5 mM EDTA

T4-DNA Ligase buffer (10x)

50 mM Tris-HCl; 10 mM $MgCl_2$; 1 mM ATP; 10 mM DTT pH 7.5

T4-polynucleotid kinase buffer (10x)

70 mM Tris-HCl; 10 mM $MgCl_2$; 5 mM DTT pH 7.6

T4-polynucleotid kinase buffer

70 mM Tris-HCl; 10 mM $MgCl_2$; 5 mM DTT pH 7.6

T4-DNA Ligase buffer

50 mM Tris-HCl; 10 mM $MgCl_2$; 1 mM ATP; 10 mM DTT pH 7.5

Exonuclease VII (Epicentre 5X Reaction Buffer: 250 mM Tris-HCl (pH 7.9); 250 mM Sodium phosphate (pH 7.8); 50 mM 2-mercaptoethanol; 42 mM EDTA.

WALKER running buffer

25 mM Tris; 192 mM glycine pH 8.3

WALKER loading dye (5x)

1.55 ml 1 M Tris-HCl pH 6.8; 250 µl 1% bromphenolblue; 700 µl A. bidest; 2.5 ml 100 % Glycerol

Chemicals

Sulfo-SMCC, No-Weigh™ Format, 8 × 2 mg microtubes; Thermo Scientific; No. 22622

dNTP's (100 mM) Peqlab, 20-1010, 20-1020, 20-1030, 20-1040

complete, Mini Protease Inhibitor Cocktail Tablets; Roche; No. 04 693 124 001

Ponceau red ; VWR, A2935.0500

SYBRGreen ; Invitrogen # S7563

Coomassie G250 ; Roth

Page Ruler Protein Ladder Plus Fermentas

100 bp ladder; NEB

Cy3 labeled dUTP; Jena Bioscience, NU-803-CY3

Biotin-16labeled dUTP; Roche, 11093070910

General chemicals: Sigma-Aldrich

Enzymes

T4-polynucleotid kinase 10,000 units/ml; NEB, M0236 S

T4-DNA Ligase 400,000 units per ml; NEB, M0202T

Exonuclease VII; Epicentre

Exonuclease VII – buffer; Epicentre

Phi29 Polymerase; NEB, M0269L

Gels

Table 1: 15 % SDS - PAA - Gel

	Separating gel (5 ml)	Stacking gel (2ml)
A. bidest	1.1 ml	1.4 ml
30 % Acrylamide	2.5 ml	0.33 ml
	1.3 ml 1.5 M Tris (pH	0.25 ml 1 M Tris (pH
10 % SDS	0.05 ml	0.02 ml
10 % APS	0.05 ml	0.02 ml
TEMED	0.005 ml	0.-002 ml

Table 2: 8 % PAA Gel (big)

	Separating gel (100 ml)
A. bidest	72.2 ml
40 % Acrylamide	17.8 ml
10 x TBE	10 ml
1,6 % APS	3.3 ml
TEMED	50 µl

Take 5 ml of total volume, add 300 µl 1.6 % APS and 30 µl TEMED for sealing the chamber

Table 3: native PAGE according to (Walker 1994)

	Separating gel (6%)	Stacking gel (5%)
A. bidest	16.35 ml	7.4 ml
30 % Acrylamide	6 ml	1.5 ml
	7.5 ml 1.5 M Tris (pH	3 ml 1 M Tris (pH 6.8)
10 % APS	150 µl	100 µl
TEMED	15 µl	2.25 µl

Table 4: Blue Native PAGE according to (Wittig, Braun et al. 2006)

	Sample gel	Separation gel
	3.5% acrylamide	12 % acrylamide
GEL-AB mix	3.31 ml	56.8 ml
Gel buffer 3x	6.6 ml	33.3 ml
Glycerol	-	20 g
water	9.15 ml	9.9 ml
Total volume	20 ml	100 ml
10% APS	200 µl	500 µl
TEMED	20 µl	50 µl

Equipment

SLIDE-A-LYZER® MINI Dialysis Units; 7,000 MWCO; Thermo Scientific; No. 69560

illustra MicroSpin G-50 Columns; Sephadex™ in TE-Buffer; GE Healthcare; No. 27-5330-01

Amicon® ULTRA Centrifugal Filters – 0.5 ml 100K; Millipore; No. UFC510008

Fluorescencescanner Fujifilm FLA 5100

Microcon Centrifugal Filter Device YM30 - 0.5 ml; 30K; Millipore No: 42409

Nitrocellulose-membrane; Sigma; #N6018

PVDF-membrane; Millipore # IPVH00010

Oligonucleotides

	sequence	specifications
P1	SH- AAA AAA AAA AAA AAA GAA TAC GCC CTC AGA AAA TC	15A linker based on paper Schweitzer 2002, Thiol-Group at 5'End, O1 binding site (red)
P2	SH-Cy3- AAA AAA AAA AAA AAA GAA TAC GCC CTC AGA AAA TC	SH and Cy3 at 5' end modified version of P1
P3	SH-ACAGAGTTATCCACAGTAGAT	SH at 5' end for conjugation
P4	5`-Fluoro ATCTACTGTGGATAACTCTGT	Fluorescein label at 5`-end, reverse complement to P3
	sequence	specifications
P5	Alexa546 - TAGGAGTGGGGAAGTCT	Primer binds to RCP of O1; 5'-end with Alexa546
O1	GCTCG G AC CGAAA A CCC AAC CCG CCC TAC CC A AAA GAC CGA TGG AAA C GAG CGC AT G ATT TTC TGA G GGC GTA TTC ATG C	internal binding site for P1, P2 and P5, hairpin for ligation
O2	CTCG A T T TTC TGA GGGC G ATT TTC TGA G GGC GTA TTC T CTG GAA CAG TAC CA C GAG CGC AT G GTG ATG GTG ATG ATG CG	internal binding site for P1, P2 and P5, hairpin for ligation T to C Mutation

Oligonucleotides were delivered by either TIB MOLBIOL, Berlin or Invitrogen.

2.2 Conjugation of DNA to Protein

Used Proteins for conjugation:

Protein / AB	Concentration	Buffer	Molecular weight
GST-CREB (STIP)	2 $\frac{mg}{ml}$	2xPBS, 20 mM Gluthation, 1 mM DTT	90 kDa (22.22 $\frac{pmol}{\mu l}$)
His-tagged Protein Filamin A	2 $\frac{mg}{ml}$	2xPBS and 1 mM DTT, 250 mM Imidazol	110 kDa (18.18 $\frac{pmol}{\mu l}$)
αGST-AB	5 $\frac{mg}{ml}$	150 mM NaCl; 0.02% sodium azide	144 kDa (34.7 $\frac{pmol}{\mu l}$)
αActin-AB	0.2 $\frac{mg}{ml}$	1x PBS, 0.1 % sodium azide, 0.1% gelatin	144 kDa (1.38 $\frac{pmol}{\mu l}$)
GST	2 $\frac{mg}{ml}$	2xPBS, 20 mM Gluthation, 1 mM DTT	26.5 kDa (75.47 $\frac{pmol}{\mu l}$)

The conjugation of Protein / AB was performed with P1 (Sample 1, S1) and P2 (Sample 2, S2). The negative control of the conjugation (S3) was performed with Protein / AB only. Two additional negative controls contain only the primer P1 (S4) and the primer P2 (S5) respectively.

The concentration of proteins is x $\frac{mg}{\mathrm{ml}}$. Conversion to mole based units is based on the definition: 1 kDa equals to 1 $\frac{kg}{mol}$. An IgG – AB has a molecular mass of 144 kDa (144 $\frac{kg}{mol}$). According to Formula 1 the conversion is done for all proteins / AB's and exemplary shown for the αGST – AB.

Formula 1: conversion of $\frac{mg}{\text{ml}}$ to $\frac{pmol}{\mu l}$ for αGST-AB

$$5\frac{mg}{ml} = 5 * \frac{10^{-3}}{10^{-3}}\frac{g}{L} \Leftrightarrow$$

$$5 * \frac{10^{-3}}{10^{-3}}\frac{g}{L} * \frac{1 * mol}{144 * 10^{3} g} = \frac{5}{144000}\frac{mol}{L} = 3{,}47 * 10^{-5}\frac{mol}{L} = 34{,}7\frac{pmol}{\mu l} = 34{,}7\mu M$$

Based on (Soderberg, Gullberg et al. 2006) 40 µg (277pmol) AB were conjugated with 30 times more Sulfo–SMCC (8 nmol) to 2 times more primer (600 pmol) per conjugation assay. For the preliminary tests are based on the assumption of an equal distribution of free primary amins on the amino acids of the protein. If calculations are based molar ratios of protein to Sulfo-SMCC one would need the exact number of free amins per protein. The assay of (Soderberg, Gullberg et al. 2006) is not adopted to molar ratio of protein to Sulfo–SMCC to primer.

2.2.1 Dialyze Protein / antibody in SLIDE-A-LYZER against 1x PBS

If the Protein / AB contains azide or other free amin containing substances, they have to be removed prior to the conjugation by dialysis.

For every sample 40 µg Protein / AB were loaded to one SLIDE-A-LYZER. The same volume of 1x PBS was loaded to a second SLIDE-A-LYZER for the protein free controls.

The protein / AB was dialyzed in SLIDE-A-LYZER over night at 4°C against 1x PBS, while low speed stirring.

Concentration with an Amicon® ULTRA Centrifugal Filter was done to achieve a minimum protein concentration for conjugating of at least 2 $\frac{mg}{\text{ml}}$.

2.2.2 Concentration of antibody-DNA

10 µl aliquots of protein containing samples were taken for later comparison on gels.

The diluted samples were load to Amicon® ULTRA Centrifugal Filters and centrifuged at 14,000 x g at 4 °C until defined FT is left, resulting in a protein concentration of at least $2\frac{mg}{\mathrm{ml}}$ in the retentate. The FT's were kept for checking a possible loss of proteins on gel. The sample reservoir is placed upside down in a new vial and centrifuged for 2 min at 1000 x g.

2.2.3 Buffer exchange for protein / antibody

G-50 gel columns were equilibrated with P-buffer (one for each sample: S1, S2, S3, S4 and S5), therefore the columns were vortexed to resuspend the resin and cap and bottom closure were loosened. G-50's were placed in supplied collection tubes and spun 1 min at 735 x g for the removal of storage TE-buffer solution. 50 µl P-buffer were load to G-50's and centrifuged 1 min at 735 x g for the buffer exchange. The collection tubes were discarded and replaced. The buffer exchange of the Protein / AB was done for samples S1, S2 and S3 (Protein / AB - Sulfo-SMCC) and for the controls S4 and S5 containing no protein. The samples were loaded to the columns and centrifuged for 2 min at 735 x g. The sample containing FT's were kept on ice, while the G-50's were placed in new collection tubes washed twice with 50 µl p-buffer. Therefore p-buffer was loaded to each G50 and centrifuged 1 min at 2000 x g. The FT's (W1) were kept and collection tubes replaced. 50 µl p-buffer were loaded again to G50's and centrifuged 1 min at 735 x g. The FT's (W2) were kept on ice, while collection tubes were replaced. For the second buffer exchange of the protein containing samples (and controls without protein), samples were load to the appropriate washed G-50's and centrifuged 2 min at 735 x g. The sample containing FT's were kept on ice and the columns were washed a third time with 50 µl p-buffer while spinning at 735 g for 1 min. The FT's were kept as W3.

2.2.4 Mix conjugation reagent and Protein / antibody

2 mg Sulfo-SMCC in were dissolved in 145.8 µl DMSO and transferred to 1000 µl P-Buffer. 2 µl (8 nmol) of the 4 mM Sulfo-SMCC were added to each sample of buffer exchanged Protein / AB (40 µg) and no protein containing controls. The samples were incubated for 2 hours at 25 °C.

2.2.5 Buffer exchange to remove unbound Sulfo-SMCC from protein / antibody

The separation of unbound Sulfo-SMCC was done for each sample like Buffer exchange for Protein / AB in G-50 spin columns (G-50) against P-buffer. Only the taken aliquots of washing steps were named sW1, sW2 and sW3, instead of W1, W2 and W3.

2.2.6 Primer preparation

2.2 nmol primer P1 were dissolved in 22 µl A. bidest to set primer to 100 µM. Also primer primer P2 was set to 100 µM. For each conjugation 600 pmol primer (P1/P1) were prepared for S1 / S2 and S4/S5. 277 pmol AB were incubated with 600 pmol P1 (100 µM) per assay S1 and S3 and 600 pmol P2 per assay S2 and S5 resp. Primers were reduced for the single assays according to Table 5.

Table 5: Reducing primer 1 and primer 2 with DTT prior to conjugation

	S1	S4	S2	S5	S3 (2 times)
P1 (100µM)	6 µl	6 µl	-	-	-
P2 (100µM)	-	-	6 µl	6 µl	-
DTT (100 mM)	8 µl	8 µl	8 µl	8 µl	8 µl
P-buffer	36 µl	36 µl	36 µl	36 µl	36 µl
Total	50 µl	50 µl	50 µl	50 µl	50 µl

Remove of the DTT a buffer exchange with new G-50's was performed. The separation of DTT from primer was done for each sample like buffer exchange for Protein / AB in G-50 spin columns (G-50) against P-buffer. Only the taken aliquots of washing steps were named oW1, oW2 and oW3, instead of W1, W2 and W3.

2.2.7 Mix samples with primers

The Protein / AB-solutions and the primer solutions were diluted. The Protein / AB solutions were in 50 µl P-Buffer. The 50 µl from each reduction of the primer were now in 55 µl P-Buffer. The appropriate Protein / AB solutions (+ Sulfo–SMCC) and the primer containing samples were mixed according to Table 6.

Table 6: Mixing scheme for Protein / AB and primer P1 / P2

Sample	Protein	Reduced primer	Volume before	Volume after dialysis
	[µl]	[µl]	[µl]	[µl]
S 1 (Protein / AB + P1)	50	55	105	110
S 2 (Protein / AB + P2)	50	55	105	110
S3 (Protein / AB only)	50	55	105	150
S4 (P1 only)	50	55	105	105
S5 (P2 only)	50	55	105	105

Mixed compounds were incubated for 2h's at 4 °C.

2.2.8 Dialysis of antibody-DNA conjugate

Dialysis of the conjugation assays were performed with 30 ml PBS (+ 5 mM EDTA at pH 7.2) over night at 4 °C using the SLIDE-A-LYZER in floating device at low speed setting stirring.

2.2.9 Validation of conjugation

Samples of the conjugation as well as from the washing steps were load to the appropriate PAA-gels documented in 3.1 (page 38 to 56) All samples were incubated for 5 min at 95°C before loading to gel and the electrophoresis was done for ~1h at 120 V. The protein containing gels were scanned first for Cy-3 signals with Fluorescence scanner (λ_{em} 532 nm) and stained afterwards with Coomassie. The DNA containing gels for the FT's of the primer reduction were scanned for Cy3–signals and stained with SYBRGreen for visualizing the DNA in a fluorescence scan.

2.2.10 Concentrate antibody-DNA

10 µl aliquots of conjugated samples were taken for later comparison of the concentration process on SDS-PAGE. The Protein / AB - DNA samples were load to Amicon® ULTRA Centrifugal Filters and centrifuged at 14.000 x g at 4 °C until defined filtrate is left. The FT's were kept for controlling the loss of protein. Placing the sample reservoirs upside down in a new vial and spinning them for 2 min at 1000 x g was done to transfer concentrated retentate to vial. The aliquots of the concentration process were checked on 15 % SDS PAA-Gel.

2.2.11 Validate conjugation of P1 / P2 on antibody

Samples were mixed with 2 µl CCE-buffer in 12 µl total volume, heated up for 5 min at 95°C and loaded to 15 % SDS-PAA-Gel. Electrophoresis was performed for ~1h at 120 V. Incubation of the gel in Fixation buffer (50% EtOH + 5% Acetic Acid) over night and subsequent washing four times with A. bidest for 45 min each was performed until gel gained the original size. A 45 min incubation step in silver solution (10 ml Stock to 140 ml A. bidest) was followed by rinsing the gel in A. bidest for 30 sec. The gel was placed in reducer solution (ready made in equal parts of reducer aldehyde and reducer base) for 10 min. The reducer solution was made in equal parts of Reducer Aldehyde Working solution (10 ml in 65 ml A. bidest) and Reducer Base Working solution (10 ml in 35 ml A. bidest). Rinsing the gel in water for 15 sec was followed by a 60 min incubation of the gel in stabilizer solution (10 ml in 440 ml A. bidest). The gel is scanned using a color scanner.

2.2.12 Validate conjugation of P1 / P2 on antibody with WALKER native PAGE

Cast the native gels according to (Walker 1994) composed as listed in Table 3. Mix the proteins with WALKER loading dye (5x) and load to gel. Use the WALKER running buffer to run the gel at 20 mA for approx. 3h. Scan with color scanner.

2.2.13 Validate conjugation of P1 / P2 on antibody with Blue Native PAGE

Rotiphorese® Gel A: 30 % Acrylamid

Rotiphorese® Gel B: 2 % Bisacrylamid

Using the above mentioned acrylamide solutions one can vary the size of pores in the PAA-gel to the given specifications. The formula:

$$V_a = \frac{T \text{ x } (100 - C) \text{ x } V_t}{3000} \quad V_b = \frac{T \text{ x } C \text{ x } V_t}{200}$$

Formula 2: Calculation of the appropriate ratios of Gel A and Gel B, to produce gels with determined C and T values.

V_t = Total volume of gelsolution (ml)

T = concetration of gel in % = % Acrylamide + % Bisacrylamide

C = Crosslinking in % = (% Bisacrylamide x 100) / T

V_a = Volume Gel A in ml

V_b = Volume Gel B in ml

12 % PAA-separationgel with a C-value of 3 is used to separate the 144 kDa AB from the nucleotide labeled one.

$$V_a = \frac{12 \text{ x } (100 - 3) \text{ x } 100 \text{ ml}}{3000} = 38{,}8 \text{ ml}$$ Gel A is mixed with $$V_b = \frac{12 \text{ x } 3 \text{ x } 100 \text{ ml}}{200} = 18 \text{ ml}$$ Gel B. Total Volume of AB-mix is 56.8 ml.

The 10% and 8 % PAA gels were cast using the same Formula 2.

For the 3.5 % sample gel:

$$V_a = \frac{3{,}5 \text{ x } (100 - 3) \text{ x } 20 \text{ ml}}{3000} = 2{,}26 \text{ ml}$$ Gel A is mixed with

$$V_b = \frac{3{,}5 \text{ x } 3 \text{ x } 20 \text{ ml}}{200} = 1{,}05 \text{ ml}$$ Gel B to a total Volume of AB-mix 3.31 ml.

The 10% and 8% PAA-Gel with a C-value of 3 for Blue Native Page were casted the

same way using the above mentioned formula.

Anode and cathode buffer were load to gel chamber and protein samples were set to 10 µl with A. bidest and 2 µl 100 % Glycerol were added. Electrophoresis was performed at 4°C, while 11 mA and 25 V per cm were applied. Electrophoresis was paused when the gel front did reach one third of the total gel length. The cathode buffer was changed to cathode buffer B/10 and electrophoresis was continued. Destaining the gel was done in water and gel is documented using a color scanner.

Western blot (WB)

PVDF membrane and nitrocellulose membrane were used to perform WB. If a PVDF membrane was used it needed to be activated in 70 % EtOH. WB was performed with 3 pieces of blotting buffer soaked Whatman® paper on Kathode. The gel was placed on Whatman® paper and the membrane was placed above. Six pieces of blotting buffer soaked Whatman® paper were placed over membrane for separation from the anode. Semidry blot (Biorad) was run with 0.8 mA per cm^2 for 60 minutes. The membrane was washed for 1 min with A. bidest and stained with Ponceau red for 5 min, to check if proteins are visible and therefore transferred to the membrane. Destaining of the Ponceau red was done with TBS-T until red color was gone. The gel for WB was stained with Coomassie for validating the transfer performance. Membrane was blocked over night in 3 % BSA containing PBS while shaking and washed for 5 x 5 min with 5 ml PBS + 0.05% Tween. The incubation of the blocked membrane with the 1st AB (diluted 1 to 1000 with 1 % BSA–PBS) was done for 90 min while shaking. The membrane with 1st AB was washed 2 x 2 min with PBS + 0.05% Tween. The 2nd AB was applied to membrane (dilute 2nd AB 1 to 1000 with 1 % BSA-PBS) and incubated for 60 min while shaking. The membrane was washed 2 x 2 min in PBS + 0.05 % Tween. According to label of the 2nd AB the membrane was scanned at fluorescence scanner at the appropriate wavelengths.

Cell lysis protocol

Samples were collected with 1.5 ml of cells after passaging. Cells were centrifuged to a pellet at 4 °C for 30 s at 6000 x g. Medium was aspirated before cells were washed with 1 ml of chilled PBE. Centrifugation and washing steps were repeated 5 times. 1.5 ml of chilled PBS were pipetted into the tube to resuspend the pellet and transferred to new tube. Centrifuge tubes were placed on ice until the chilled tube was centrifuged at 6000 x g for 30 s at 4° C. The supernatant was removed and the pellet was placed on ice. The cell lysis was performed using pink buffer (PB), which was set to room temperature as well as the cell pellet. No PBS was left in the cell pellet, before the cell pellet was resolved in 50 µl of PB. After the cell pellet was dissolved the lysis was performed on ice until no pellet remains visible. Centrifugation of the cell lysate was performed at 16000 x g for 30 min at 4 °C to spin down cell debris. The protein containing supernatant was stored at 4°C.

2.3 Rolling circle amplification

2.3.1 Generation of the linear template

Phosphorylation of linear template oligonucleotide (O1 / O2)

Phosphorylation assays were performed to phospohorylate the 5' and of the linear template oligonucleotide. The negative control is performed without DNA.

Table 7: Phosphorylation assay

	O1 / O2	O1 / O2-NC
100 µM O1 / O2	8 µl (800 pmol = 8 µM)	-
T4-polynucleotid kinase	8 µl (40 U)	-
ATP 100 mM	12 µl (10 mM)	8 µl (160 U)
T4-polynucleotid kinase buffer	12 µl	12 µl (10 mM)
A. bidest	80 µl	12 µl
total	120 µl	88 µl

Everything was mixed on ice. The oligonucleotide was heated up for 5 min at 95° C, for disruption of the secondary structure and transferred to ice cold phosphorylation assay before adding the T4-polynucleotid kinase. The assays according to Table 7 were incubated for 30 min at 37 °C and inactivated at 75 °C for 15 min.

Hybridization of template DNA O1 / O2

To get the secondary structure of the hairpin oligonucleotide O1 / O2, a hybridization step at 34°C was performed. The predicted T_M for O1 is 34°C.

The linear template oligonucleotide (O1 / O2) was heated up to 75°C for 15 min and instantly cooled down and incubated over night at 25°C.

12 µl aliquot (80 pmol) were taken after phosphorylation (aP): aP-O1 and aP-O1-NC.

720 pmol O1 / O2 were left in 108 µl.

Ligation of linear O1 / O2

Table 8: ligation assay for the phosphorylationassays of O1 / O2

	Volume	Volume-NC
Phosphorylationassay	108 µl (720 pmol O1 / O2)	-
Phosphorylationassay-NC	-	96 µl
T4-DNA ligase	1 µl (200 units)	1 µl (400 units)
T4-DNA ligase buffer	13 µl (1 mM)	16 µl (1 mM)
A. bidest	8 µl	47 µl
total	130 µl	160 µl

Ligation assay in table 7 were incubated over night at 16 °C and deactivated for 15 min at 75°C before setting to RT (25°C).

14.45 µl aliquot (80 pmol) after ligation (aL): aL- O1/O2and aL - O1/O2-NC were taken to evaluate ligation performance on gel.

Two times 640 pmol O1 / O2 are left in 118 µl each. (5.43 µM)

Validate Ligation – Exonuclease VII restriction

Exonuclease VII assay was incubated for 2 h at 37 °C and deactivated for 10 min at 95 °C. The assay was stored at 4 °C.

Table 9: Exonuclease VII assay for Exo7

	Volume – O1 / O2 (2x)	Volume- O1 / O2-NC
O1/O2– ligationassay	22.1 µl (120 pmol)	-
O1/O2– ligationassay-NC	-	44.2 µl
Exonuclease VII (10 u per µl)	3 µl (30 U)	6 µl (60 U)
Epicentre buffer (5x)	12 µl	24 µl
A. bidest	22.9 µl	45.8 µl
total	60 µl	120 µl

Sum up for the generation of the circular RCA – template:

The 120 µl starting volume contains 800 pmol O1 / O2. The 12 µl sample for the phosphorylation – step contains 80 pmol O1 / O2. 108 µl phosphorylated O1 / O2 are refilled to 130 µl for ligation and contain 720 pmol O1 / O2. After Ligation 14.45 µl sample are taken containing 80 pmol circular O1 / O2. 115.55 µl containing 640 pmol O1 / O2 (5.43 µM) are left.

For Exonuclease VII restriction 22.1 µl (120 pmol) of ligation assay are used.

120 pmol O1 / O2 are in 60 µl of exonucleation assay. The circular template for the RCA is 2 µM.

Check ligation performance on 8 % PAA-Gel (big gel).

Load the samples with 2 µl 100 % Glycerol, set to 12 µl with A. bidest and perform electrophoresis at 285 V for 2-2.5 h (see figure on page 52).

2.3.2 RCA on generated template

Hybridization of O1 with P1 / P2

The RCA needs a starting point, which is the hybridized primer on the circular template. Therefore the generated circular template was hybridized to the primer. The following samples were taken:

1. Hybridize 12.5 µl (25 pmol) of O1 (2 pmol/µl) with 0.5 µl (50 pmol) of P1 (100 µM).

1a. Hybridize 12.5 µl (25 pmol) of O1 (2 pmol/µl) with 0.5 µl (50 pmol) of P2 (100 µM).

1-NC. Hybridize 12.5 µl of O1-NC with 0.5 µl (50 pmol) of P2 (100 µM).

All assays were incubated for 15 min at 75°C and incubated over night at 25°C.

RCA of O1 with P1 / P2

The RCA is performed on hybridized Exonucluase VII treated circular template O1 and the primer P1 / P2 in 36 µl assays.

Table 10: RCA assay for Exonuclease VII treated circular template O1 and primer P1 / P2

	Volume P1	Volume P1-Cy3	Volume-NC of RCA
O1-P1 (1)	13 µl	-	-
O1-P2 (1a)	-	13 µl	-
O1-NC-P2 (1-NC)	-	-	13 µl
dATP, dCTP, dGTP, dTTP	1 µl of 100 mM each	1 µl of 100 mM each	1 µl of 100 mM each
Phi29 (50 Units, 10,000 U per ml)	5 µl	5 µl	5 µl
Phi29-buffer	3.6 µl	3.6 µl	3.6 µl
BSA (10 mg/ml)	0.8 µl	0.8 µl	0.8 µl
A. bidest	9.6 µl	9.6 µl	9.6 µl
total	36 µl	36 µl	36 µl

10 µl aliquot were taken from each RCA-assay and deactivated for 20 min at 70 °C. Incubate the RCA-assay for 180 min at 30°C and inactivate 20 min at 70°C. Store at 4°C.

Validate RCA via 8 % Polyacrylamidgel (big)

5 µl of every RCA-sample (before and after the RCA was performed) were set to 10 µl with A. bidest and 2 µl 100 % Glycerol were added. Load 12 µl of the RCA-

samples to 8 % polyacrylamidgel gel. Use a 100 bp DNA ladder and the Page Ruler protein ladder. Load 0.5 µl of 100 µM O1, 1 µl 100 µM P1 and 0.125 µl of 100 µM P2 as controls.

2.4 Rolling circle amplification on Antibody

RCA of template O1 with primers P1 / P2 on AB

The assays contains Exonuclease VII treated circular template O1.

The hybridization of O1 with αGST-AB-P1 / αGST-AB-P2 was performed with the circular template O1, heated up for 5 min at 75 °C for a better hybridization effect, and the conjugated primers P1 / P2.

2. Hybridize 12.5 µl (25 pmol) of O1 with 7.5 µl of αGST-AB-P1. 3.75 pmol αGST-AB-P1 in 20 µl are 0.18 µM αGST-AB and 1.25 µM O1.

2-NC. Hybridize 12.5 µl (0 pmol) of O1-NC with 7.5 µl of αGST-AB-P1. 3.75 pmol αGST-AB-P1 in 20 µl are 0.18 µM αGST-AB.

3. Hybridize 12.5 µl (25 pmol) of O1 with 7.5 µl of αGST-AB-P1. 3.75 pmol αGST-AB-P1 in 20 µl are 0.18 µM αGST-AB and 1.25 µM O1.

3-NC. Hybridize 12.5 µl (0 pmol) of O1-NC with 7.5 µl of αGST-AB-P2. 3.75 pmol αGST-AB-P2 in 20 µl are 0.18 µM αGST-AB.

4. Hybridize 12.5 µl (25 pmol) of O1 with 7.5 µl of αGST-AB. 3.75 pmol αGST-AB (S3) in 20 are 0.18 µM αGST-AB and 1.25 µM O1.

4-NC. Hybridize 12.5 µl (0 pmol) of O1-NC with 7.5 µl of αGST-AB. 3.75 pmol αGST-AB (S3) in 20 µl are 0.18 µM αGST-AB.

5. Hybridize 12.5 µl (25 pmol) of O1 with 7.5 µl of αGST-AB (S4).

5-NC. Hybridize 12.5 µl (0 pmol) of O1-NC with 7.5 µl (0 pmol) of αGST-AB (S4).

6. Hybridize 12.5 µl (25 pmol) of O1 with 7.5 µl of αGST-AB.

6-NC. Hybridize 12.5 µl (0 pmol) of O1-NC with 7.5 µl of αGST-AB (NC2).

Incubate samples 2 to 6 (including NC's) for 15 min at 37°C and over night at 25°C.

RCA of O1 with samples 2 to 6 (including NC's)

The hybridized assay from above were used for the RCA according to Table 11

Table 11: RCA assay for hybridized AB containing samples.

	Volume	Volume-NC
Hybridized assay containing O1 (2-6)	20 µl	-
Hybridized assay containing O1-NC (2-NC - 6-NC)	-	20 µl
dATP, dCTP, dGTP, dTTP	1 µl of 100 mM each	1 µl of 100 mM each
Phi29 (50 Units, 10,000 U per ml)	5 µl	5 µl
Phi29-buffer	3.6 µl	3.6 µl
BSA (10 mg/ml)	0.8 µl	0.8 µl
A. bidest	2.6 µl	2.6 µl
Total	36 µl	36 µl

Take 5 µl sample each and deactivate 20 min at 70 °C. Incubate RCA-assay for 180 min at 30°C and inactivate 20 min at 70°C. Store at 4°C.

Validate RCA on AB via 8 % Polyacrylamidgel (big)

Every sample (2 - 6) was set to 10 µl with A. bidest and 2 µl 100 % Glycerol were added. 5 µl of the RCA-Products are loaded to 8 % polyacrylamidgel gel with 2 µl 100 % Glycerol and 5 µl A. bidest. A 100 bp DNA ladder and the Page Ruler protein ladder were also load to the gel, as well as 0.5 µl of 100 µM O1, 1 µl 100 µM P1 and 0.125 µl of 100 µM P1 as controls.

RCA of O1 with P1 / P2 on antibody using modified dNTP's

All assays were incubated for 15 min at 75°C and hold over night at 25°C.

Hybridization of circular template O1 with P1 / P2

1. Hybridize 12.5 µl (25 pmol) of O1 (2 pmol/µl) with 0.5 µl (50 pmol) of P1 (100 µM).

(3x: For assay with normal dTTP and fluorescent probe

For assay with Cy3-dUTP

For assay with biotin - dUTP + later Cy3-STAV

1-NC. Hybridize 12.5 µl of O1-NC with 0.5 µl (50 pmol) of P2 (100 µM).

Hybridization of O1 with αGST-AB-P1 / αGST-AB-P2

2. Hybridize 25 µl (50 pmol) of O1 with 15 µl (7.5 pmol) of αGST-AB-P1. αGST-AB-P1 (S1) in 40 µl is 0.1875 µM. For RCA on GST membrane (3x).

2A. Hybridize 12.5 µl (25 pmol) of O1 with 7.5 µl (3.75 pmol) of αGST-AB-P1. αGST-AB-P1 (S1)in 20 µl is 0.1875 µM. For RCA in solution.

3. Hybridize 25 µl (50 pmol) of O1 with 15 µl (7.5 pmol) of αGST-AB-P2. αGST-AB-P2 (S2) in 40 µl is 0.1875 µM. For RCA on GST membrane (3x).

3A. Hybridize 12.5 µl (25 pmol) of O1 with 7.5 µl (3.75 pmol) of αGST-AB-P2. αGST-AB-P2 (S2) in 20 µl is 0.1875 µM. For RCA in solution.

4. Hybridize 25 µl (50 pmol) of O1 with 15 µl (7.5 pmol) of αGST-AB. αGST-AB (S3) in 40 µl is 0.1875 µM. For RCA on GST membrane (3x).

4A. Hybridize 12.5 µl (25 pmol) of O1 with 7.5 µl (3.75 pmol) of αGST-AB (S3). αGST-AB (S3) in 20 µl is 0.1875 µM. For RCA in solution.

5. Hybridize 25 µl (50 pmol) of O1 with 15 µl of αGST-AB (S4). For RCA on GST membrane (3x).

6. Hybridize 25 µl (50 pmol) of O1 with 15 µl of αGST-AB (S5). For RCA on GST membrane (3x).

The AB is further diluted. From 1 : 70 (34.7 $\frac{pmol}{\mu l}$ to 0.5 $\frac{pmol}{\mu l}$) during conjugation process additional dilution during hybridization to circular template O1 (0.5 $\frac{pmol}{\mu l}$*15 µl=7.5 pmol in 40 µl ; 0.1875 $\frac{pmol}{\mu l}$ for AB - DNA).

The hybridized assays from above were used for the RCA according to Table 12 and Table 13.

Table 12: RCA with modified dUTP's

	Volume-Cy3-dUTP	Volume-biotin-dUTP	Volume normal dTTP	Volume NC
Hybridized O1 and P1 (1)	13 µl	13 µl	13 µl	-
Hybridized O1-NC and P1 (1-NC)	-	-	-	13 µl (NC)
dATP, dCTP, dGTP	1 µl of 100 mM each	1 µl of 100 mM each	1 µl of 100 mM each	1 µl of 100 mM each
dTTP	0.2 µl (100 mM)	0.2 µl (100 mM)	1 µl (100 mM)	1 µl (100 mM)
dUTP-Cy3	1 µl (1 mM)	-	-	-
dUTP-biotin	-	1 µl (1 mM)	-	-
Phi29 (70 Units, 10,000 U per ml)	5 µl	5 µl	5 µl	5 µl
Phi29-buffer	3.6 µl	3.6 µl	3.6 µl	3.6 µl
BSA (10 mg/ml)	0.8 µl	0.8 µl	0.8 µl	0.8 µl
A. bidest	9.4 µl	9.4 µl	9.6 µl	9.6 µl
total	36 µl	36 µl	36 µl	36 µl

5 µl of every assay for before RCA were taken. The RCA-assays were incubated at 180 min at 30°C and inactivated for 20 min at 70°C.

Table 13: RCA with Cy-3-dUTP

	Volume-Cy3-dUTP
Hybridized assays 1A, 2A and 3A	20 µl
dATP, dCTP, dGTP	1 µl of 100 mM each
dTTP	0.2 µl (100 mM)
dUTP-Cy3	1 µl (1 mM)
Phi29 (70 Units, 10,000 U per ml)	5 µl
Phi29-buffer	3.6 µl
BSA (10 mg/ml)	0.8 µl
A. bidest	2.4 µl
Total	36 µl

5 µl of every assay for before RCA were taken. The RCA-assays were incubated at 180 min at 30°C and inactivated for 20 min at 70°C.

2.5 Validation of the αGST – AB after conjugation

Immobilize purified GST via Western Blot

Different amounts of GST were applied to 15% PAA-Gel and transferred with electrophoresis to nitrocellulose membrane.

GST-tag has 26.5 kDa and is 2 $\frac{mg}{ml}$ = 2 $\frac{\mu g}{\mu l}$; 26.5 kDa= $26{,}5\frac{kg}{mol}$;

$$2*\frac{10^{-3}}{10^{-3}}\frac{g}{L}*\frac{1*mol}{26{,}5*10^{3}g}=\frac{2}{26500}\frac{mol}{L}=7{,}5*10^{-5}\frac{mol}{L}=75{,}47\mu M=75{,}47\frac{pmol}{\mu l}$$

For 1000 ng: GST-tag was diluted 1:20 with PBS=GST-1 (100$\frac{ng}{\mu l}$) (30 µg in 30 µl; take 15 µl αGST-AB and 285 µl PBS) 50 µl CCE Buffer were added

For 100 ng: GST-tag was diluted 1:200 with PBS=GST-2 (10$\frac{ng}{\mu l}$) (4 µg in 200 µl; take 2 µl αGST-AB and 8 µl PBS for further dilution with 390 µl PBS. 66 µl CCE Buffer were added.

For 10 ng: GST-tag was diluted 1:2.000 with PBS=GST-3 (1 $\frac{ng}{\mu l}$) 0.2 µl were taken and mixed with 9.8 µl PBS and further diluted with 390 µl PBS add 66 µl CCE Buffer.

For 1 ng: GST-tag was diluted 1:20.000 with PBS=GST-4 (0.1 $\frac{ng}{\mu l}$) 40 µl GST-3 were mixed with 360 µl PBS add 66 µl CCE Buffer were added.

Table 14: Loading scheme for 15 % SDS-PAA WB of GST for GST detection with αGST-AB and either 2nd AB or RCA on AB (repeat this for the 24 WB's)

Lane	1	2	3	4	5
	Page Ruler	1 µg GST	100 ng GST	10 ng GST	1 ng GST
		12 µl GST-1	12 µl GST-2	12 µl GST-3	12 µl GST-4
		45.27 pmol	4.53 pmol	0.45 pmol	0.045 pmol

According to Table 14 50.3 pmol GST were on each membrane. Perform WB as described on page 25. The incubation with the 1st AB varieties according as described below.

Detection of the αGST-AB after conjugation

The pre - hybridized αGST-AB–P1 / P2 from page 32 (assays 2 - 6) were used as 1st AB for detection of the immobilized GST

For detection with 2nd AB (αDonkey AB–Cy 5)

PK (0.9 µl (33 pmol) αGST-AB in 3 ml 1 % BSA - PBS) [PK]

αGST-AB-P1 (7.5 µl (3.75 pmol) in 3 ml 1% BSA - PBS) [S1]

αGST-AB-P2 (7.5 µl (3.75 pmol) in 3 ml 1% BSA - PBS) [S2]

αGST-AB (7.5 µl (3.75 pmol) in 3 ml 1% BSA - PBS) [S3]

P1 (7.5 µl in 3 ml 1% BSA - PBS) [S4]

P2 (7.5 µl in 3 ml 1% BSA - PBS) [S5]

Each membrane was incubated for 90 min with 1st AB while shaking. The incubation of each membrane with 3 µl αDonkey-AB with Cy5 label was done in 3 ml 1 % BSA – PBS for 60 min while shaking in the dark. Blots were scanned on fluorescence scanner for Cy5 signal of the 2nd AB.

RCA with αGST-antibody–DNA on membrane

The prehybridized DNA–AB was used to detect the GST and subsequent RCA was performed on membrane. A petridish was used resulting in a constant humidity during the RCA on solution. The following hybrids were used as 1st AB's:

For RCA on AB, Cy3-dUTP-RCA on AB and biotin-dUTP-RCA on AB

1. PK (0.9 µl (33 pmol) αGST-AB in 3 ml 1 % BSA - PBS) [PK]

2. αGST-AB-P1 (40 µl Hybrid (12.5 pmol S1) in 3 ml 1% BSA - PBS) [S1]

3. αGST-AB-P2 (40 µl Hybrid in 3 ml 1% BSA - PBS) [S2]

4. αGST-AB (40 µl Hybrid in 3 ml 1% BSA - PBS) [S3]

5. P1 (40 µl in 3 ml 1% BSA - PBS) [S4]

6. P2 (40 µl in 3 ml 1% BSA - PBS) [S5]

The membranes were incubated for 90 min with 1st AB while shaking. Incubation of each membrane with the RCA mix according to

Table 15, 16 and 17 was done in wet chambers for 3 h's at 30 °C.

Table 15: RCA mix for membrane bound RCA using normal dTTPs

	Volume normal-dTTP
dATP, dCTP, dGTP, dTTP	1.38 µl of 100 mM each
Phi29 (70 Units, 10,000 U per ml)	7 µl
Phi29-buffer	5 µl
BSA (10 mg/ml)	1.1 µl
A. bidest	31.38 µl
total	50 µl

Table 16: RCA mix for membrane bound RCA using Cy3-dUTPs

	Volume Cy3-dUTP
dATP, dCTP, dGTP	1.38 µl of 100 mM each
dTTP	0.28 µl (100 mM)
dUTP-Cy3	1.38 µl (1 mM)
Phi29 (70 Units, 10,000 U per ml)	7 µl
Phi29-buffer	5 µl
BSA (10 mg/ml)	1.1 µl
A. bidest	31.06 µl
total	50 µl

Table 17: RCA mix for membrane bound RCA using biotin-dUTPs

	Volume biotin-dUTP
dATP, dCTP, dGTP	1.38 µl of 100 mM each
dTTP	0.28 µl (100 mM)
dUTP-biotin	1.38 µl (1 mM) (is 27.6 µM)
Phi29 (70 Units, 10,000 U per ml)	7 µl
Phi29-buffer	5 µl
BSA (10 mg/ml)	1.1 µl
A. bidest	31.06 µl
total	50 µl

All membranes were washed 5 min with 10 ml PBS + 0.05% Tween. And scanned at the fluorescence scanner at the appropriate wavelengths.

Cy3-STAV is about 77 kDa = 77 kg / mol. One vial contains 1 mg per ml resulting in 12.99 µM.

If 50 pmol of aGST AB bound to 50 pmol GST on membrane and the RCP contains 100 dUTP-biotins (50.000 dUTP-biotins) mix 10 µl Cy3-STAV with 40 µl PBS and applied to membrane for 25 min. Membranes were washed 5 min with ml PBS + 0.05% Tween.Membranes were scanned on fluorescence scanner for Cy5 signal.

3 Results

3.1 Conjugation of DNA to Protein and Antibody

Antibodies are expensive and the quality can vary between different suppliers and even from batch to batch. Additionally the concentration of antibodies is often quiet low. Therefore the conjugation should be established with recombinant proteins and should be later transferred to antibodies. The conjugation product should lead to a shift of the corresponding protein bands, visible in Coomassie stained PAGE's and Western Blot.

3.1.1 Preliminary tests

GST-STIP

GST-STIP is a GST tagged Stress-induced-phosphoprotein 1 with a molecular mass of about 90 kDa. Conjugation of P1 should lead to a shift of about 12 kDa. The initial reaction volume of 22.85 µl contains 1x Protease Inhibitor (Roche) and 40 µg GST-STIP. The following samples are used for conjugation:

S1: PBS + 600 pmol primer P1

S2: 40 µg GST-STIP + primer P1 (600 pmol)

S3: 40 µg GST-STIP (negative control of conjugation)

Samples S2 and S3 were dialyzed against 1 x PBS and afterwards concentrated using Microcon Centrifugal Filter Device–YM30 according to the guideline. 100 µl were loaded to the Filter and concentrated until 20 µl retentate were left. Afterwards all samples were dialyzed over night against P-buffer using SLIDE-A-LYZER.

8 nmol Sulfo–SMCC as conjugation reagent was added to all three samples, according to protocol on page 21, except that Sulfo–SMCC was solved in DMSO. The thiol group of primer P1 was reduced and buffer exchange to P-buffer was done as described on page 21. Primer and protein samples were mixed for 2h and dialysed over night according to protocol on page 22. Separating unbound Sulfo-SMCC from protein was done with gel column, by doing a buffer exchange. 10 µl

aliquots of the flowthroughs (FT) and the conjugation products after the buffer exchange were taken and conjugation validated with a 12 % SDS – PAGE and by WB (1st AB αGST-AB, 2nd AB αGoat-AB-Cy3). WB was done as described on page 25. As control 10 µg (100 pmol) STIP were loaded to gel.

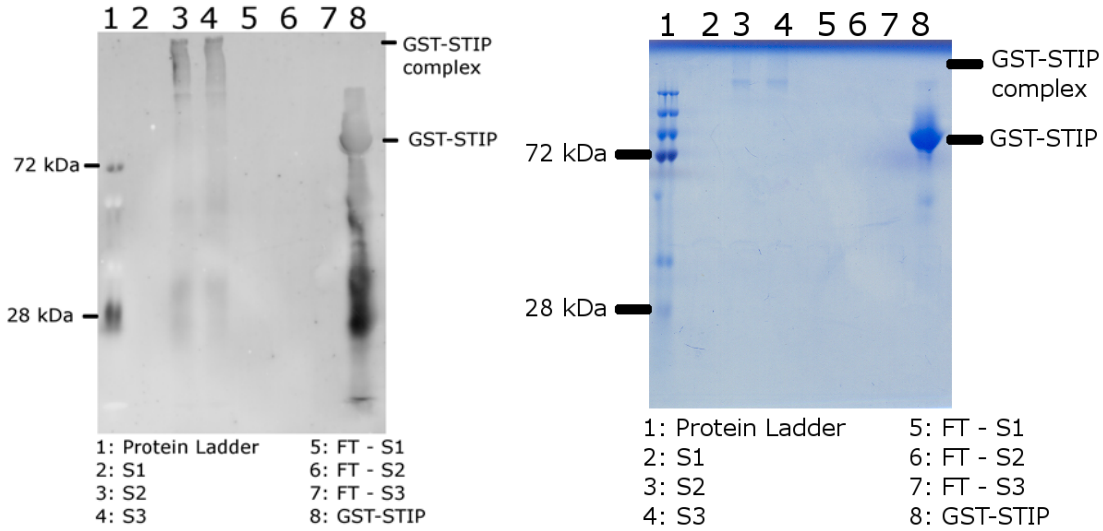


Figure 4: <u>left:</u> WB scanned at λ_{em} 532 nm for detection the GST – tagged STIP using the αGST-AB with Cy3 label. S1 (including primer P1) can be seen in lane 3 and 4 shows the negative control of the conjugation (without primer). The GST-tag (26 kDa) is visible in lane 8 as lower band. <u>right:</u> Coomassie stained gel showing the conjugated GST-STIP in comparison with the GST-STIP (lane 8). Blot and SDS-PAGE show in lanes 3 and 4 high molecular material after incubation with Sulfo-SMCC. It is very likely that this material is crosslinked GST-STIP .

The conjugation products S1 (with) and S2 (without primer) show no differences in the Coomassie stained gel and the WB (Figure 4). S1 and S2 show high molecular bands in the upper part of the gel, indicating a cross conjugation of multiple GST-STIP molecules. A specific band indicating the successful crosslink between DNA and protein cannot be detected. GST-STIP contain many free thiol groups that can be activated by Sulfo-SMCC and crosslink to any other free amine group. Additionally GST-STIP was stored in a buffer with Glutathione. Glutathione contains primary amines, which could inhibit the conjugation.

To avoid such problems the His-tagged Protein Filamin A was tested. Instead primer P1 primer P3 was used. P4, a fluorescein-labelled primer, can hybridize to P3 allowing to visualize the conjugated primer.

Protein Filamin-A

Protein 9605 is the His tagged Filamin-A protein of the human cytoskeleton with a molecular mass of about 110 kDa and a concentration of 2 $\frac{mg}{\mathrm{ml}}$ (18.18 $\frac{pmol}{\mu l}$). The conjugation was done with SH–modified primer (P3) instead of primer P1. This oligonucleotide should lead to a shift of at 8 kDa or after hybridization with P4 to a shift of approx. 16 kDa.

S1: 80 µg Filamin-A + 1200 pmol primer P3

S2: 80 µg Filamin-A + 1200 pmol hybridized P3/P4

S3: 80 µg Filamin-A (negative control of conjugation)

S4: 80 µg Filamin-A + 1200 pmol hybridized P3/P4 w/o Sulfo-SMCC

S5: 1200 pmol primer P3

1500 pmol P3

were hybridized with 1500 pmol fluorescein labeled P4. This should lead to a signal of the labeled Filamin-A in a WB. A sample (S4) without Sulfo-SMCC was prepared to study the influence of the coupling reagent on the reaction. This control was performed with 80 µg Filamin A and 1200 pmol reduced P3 / P4 and Sulfo–SMCC was replaced by 8 µl DMSO. To obtain the P3 / P4 hybrid equal amounts of both primers were mixed and incubated at 95 °C for 5 min and cooled slowly down to RT within 3 h's.

The conjugation was analyzed by SDS-PAGE and subsequent Coomassie staining for visualizing the shift directly in the gel. Additionally samples were blotted onto PVDF–membrane and incubated with αHis-AB as 1st AB and αGoat-AB-Cy3 as 2nd AB to detect His–tagged proteins. After incubation with the AB's primer P4 was hybridized against the conjugation products to detect protein bound P3 directly.

Filamin-A was prepared as described on page 19 to 23. Instead dialyzation the buffer exchange to P-buffer was done using gel columns, to avoid dilution of the Protein.

16 nmol Sulfo–SMCC was added to 80 µg Filamin-A in samples S1, S2, S3. S4 was incubated with the DMSO without Sulfo-SMCC. Unbound Sulfo-SMCC removed

according to protocol on page 21. After mixing primer and protein samples and conjugate for 2h's, samples were analyzed by SDS–PAGE. 10 µl of each conjugation product were loaded onto the gel together with 18 pmol Filamin-A as control. Additionally conjugations of all samples were validated with WB (1st AB αHis-AB, 2nd AB αGoat-AB-Cy3). WB was done as described on page 25. The membrane was scanned before blocking with a fluorescence scanner. After detection of His-tagged proteins the blot was incubated with Fluorescein labeled primer P4.

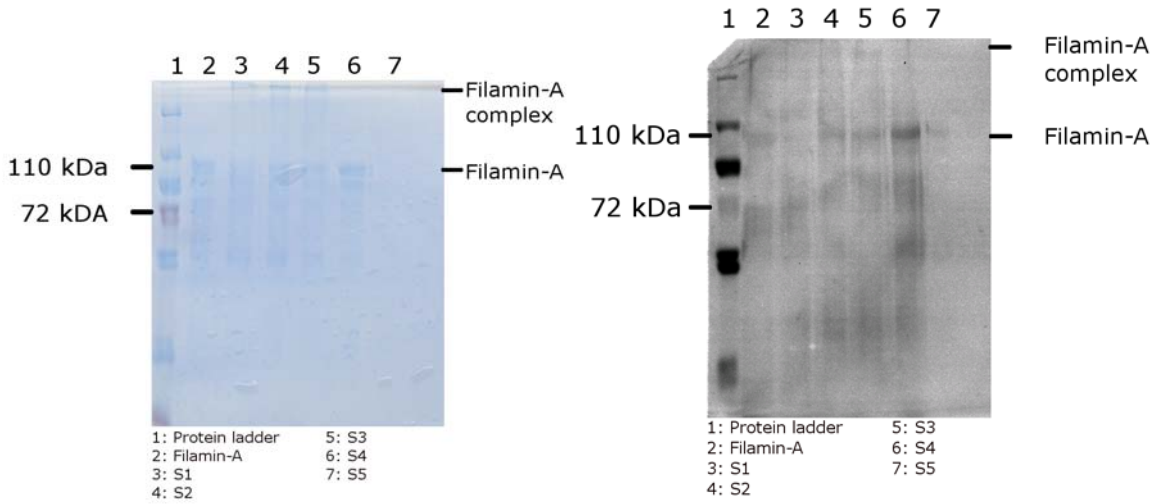


Figure 5: <u>left:</u> Coomassie stained 10 % SDS-PAGE showing the conjugation products of Filamin A. The conjugation product in lanes 3 (Filamin A + P3), 4 (Filamin A + P3 / P4) and 5 (Filamin A only) are giving the same signal in gel. The upper bands are multiple conjugates of the protein itself. In lane 6 (S4; sample w/o Sulfo-SMCC) and lane 7 (primer P3) no high molecular bands can be detected.

<u>right</u>: Fluorescence - scan of WB (λ_{em} 532nm, Cy3 signals from anti-Goat-AB) after conjugation P3 wo Filamin A. The conjugation of P3 (lane 3) and P3 / P4 hybrid (lane 4) show no differences compared to each other and to S3 (protein + Sulfo-SMCC). All 3 samples show high molecular material in the upper part of the lanes, which is not visible in lane 2 (Filamin A only) and lane 6 (Filamin A without Sulfo–SMCC). The negative control (P3 only, lane 7) gives no signal.

Figure 5 shows the conjugation products of Filamin-A in a Coomassie stained PAGE and WB. In lanes 3, 4 and 5 (samples S1, S2 and S3) high molecular bands are visible. A clear and distinct shift of the protein-DNA conjugate is not visible. All samples that contain the protein and the coupling reagent Sulfo-SMCC show these higher protein bands, meaning the high molecular material in lanes 3, 4 and 5 results from a conjugation of Filamin-A. Figure 6 shows the WB of Filamin-A and the conjugation assays S1 to S5. The signals come from the αGOAT-AB-Cy3. Cy-3 absorbs light at λ_{em} 550 nm and emits it at λ_{em} 570 nm. High molecular protein bands are also visible. The blot gives no hint if the high molecular product in the conjugation

assays contains only cross linked Filamin A or if the signal results of a multiple conjugation of DNA to Filamin A.

To address the question if the conjugation of protein to oligonucleotide P3 was successful or if the high molecular material was derived from multiple conjugations the WB was incubated with fluorescein labelled P4. P3 is only present in the lanes 3 (S1) and 4 (S2). 300 pmol P4 were incubated at 95 °C for 5 min and mixed with 3 ml PBS. The membrane was incubated over night with the PBS – P4 solution at 37 °C while shaking. The membrane was washed once with PBS – Tween (0.05 %) while shaking at 37 °C and scanned at λ_{em} 473 nm.

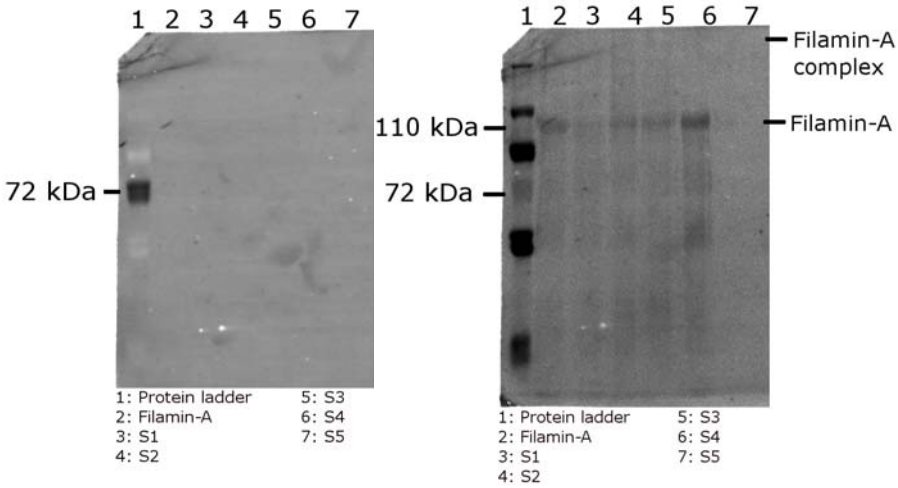


Figure 6: <u>left:</u> Fluorescence scan of the WB of Filamin A and the conjugation products after incubation with oligonucleotide P4 at λ_{em} 473 nm. Lane 4 contains the conjugation product with the P3 / P4 hybrid, lane 3 contains the conjugation product with only P3. No signal was detected. Only the Prestained Protein ladder Plus gives a signal at this wavelength. <u>right:</u> Same membrane scanned at the λ_{em} 532 nm wavelength for the Cy-3 signal of the αGOAT-AB.

Figure 6 shows the fluorescence scan after P4 incubation at two different wavelengths. No specific signal can be measured at λ_{em} 473 nm for the fluorescein labelled P4. The signals at λ 532 nm for the Cy–3 labelled αGoat-AB indicate, that Filamin A is still bound to membrane.

The answer if the conjugation product (high molecular material in lanes 3, 4 and 5 in Figure 5) was a cross conjugation of proteins or a multiple conjugation of DNA to protein could not be answered. It is likely that the complex contains mainly crosslinked protein.

It was not possible to detect a conjugation of a primer to any of the tested proteins. The next protein to test for conjugation was an AB. Soderberg *et al*. (Soderberg,

Gullberg et al. 2006) describe a protocol to visualize the conjugation product with blue native page. This method was implemented for visualization of the proteinshift.

3.1.2 Conjugation with αActin–Antibody

The αActin–AB is an IgG of about 144 kDa and a concentration of 0.2 $\frac{mg}{ml}$. The antibody was delivered in 1 x PBS and contains sodium azide and stabilizer, which were removed according to the conjugation protocol on page 18.

S1: 40 µg αActin–AB + 600 pmol primer P1

S2: 40 µg αActin–AB

S3: 600 pmol primer P1

To achieve the minimum concentration of 2 mg per ml a 1 : 10 enrichment is performed according to the protocol on page 23. The conjugation process was performed according to the protocol on page 20 and analyzed by 10 % SDS-PAGE. 10 µl of each sample were loaded onto the gel and compared with 7 pmol αActin–AB. Additional 10 µl of the flowthrough W1 to W3 (buffer exchange of αActin-AB) and sW1 to sW3 (separation of unbound Sulfo-SMCC) were analyzed.

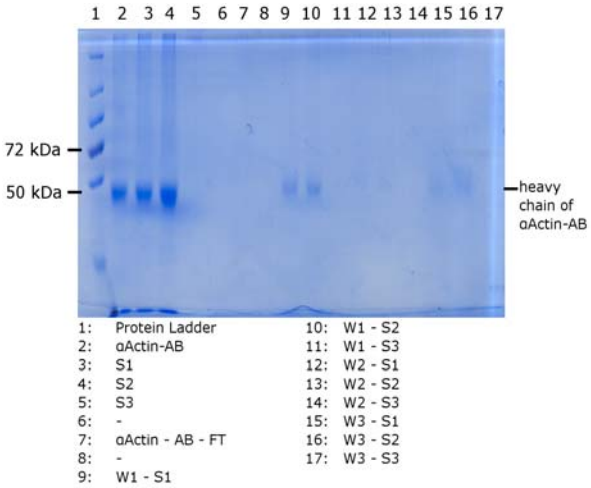


Figure 7: SDS - PAGE of αActin-AB to DNA conjugation. The Coomassie staining shows the heavy (50 kDa) chains of the αActin-AB. The light chains 24 kDa are not visible. The conjugation product of αActin-AB and the primer P1 is shown in lane 3. Lane 5 (S3) contains only the primer and gives no signal. Lane 7 shows 10 µl of the FT from AB enrichment prior to conjugation and lanes 9 to 17 the FT from the buffer exchange. The missing signals are indicating low loss of protein during the conjugation process.

Figure 7 and Figure 8 show the conjugation process of the αActin–AB. Figure 7 lane 3 shows the conjugation product with primer P1, the expected shift of the heavy chains (50 kDa) of about 12 kDa is not visible. The FT's of the washing steps W1 – W3 and sW1 – sW3 in lanes 9 to 17 shows some weak antibody bands. The loss during the washing steps is negligible.

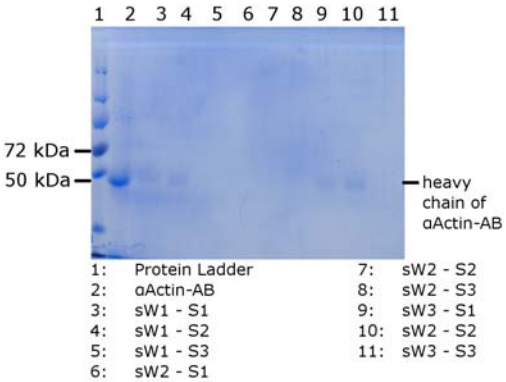


Figure 8: SDS - PAGE of αActin - AB to DNA conjugation. The SDS-PAGE shows the heavy (50 kDa) chains of the αActin - AB. The light chains 24 kDa are not visible. Lanes 3 to 11 contain the FT's after separation unbound Sulfo-SMCC from AB.

The functionality of the αActin–AB after conjugation was tested by WB. Cell lysate derived from F11 mouse-rat hybridoma cells was blotted. A Cy5 labeled αRabbit-AB was used as 2nd AB. Up to 100 µM actin can be usually found in eukaryotic cells (Wu and Pollard 2005). Cells from 10 ml cellsolution were collected and all liquid was removed. The resulting pellet (~1 µl cell; 10^7 cells) was dissolved in 50 µl Pink-buffer resulting in an actin concentration of about 2 µM (2 $\frac{pmol}{\mu l}$). The subsequent WB was performed according to the protocol on page 25 using 10 % SDS PAGE. Actin was loaded in three different concentrations ranging from 1, 10 to 30 pmol per lane. In total 41 pmol actin was loaded. Assuming an optimal transfer of the proteins the same amount of actin should be present on the membrane. Blotted actin was detected with samples S1 – S3 and unmodified antibody:

WB for PK: αActin-AB (2 µM): (3 µl in 3 ml 1 % BSA - PBS)

[6 pmol αActin–AB]

WB for S1: αActin–AB–P1 (0.75 µM) (12 µl in 3 ml % BSA - PBS)

[9 pmol αActin–AB–P1]

WB for S2: αActin-AB (1µM) (12 µl in 3 ml % BSA - PBS)

[12 pmol αActin–AB]

WB for S3: 12 µl in 3 ml % BSA - PBS)

[containing no αActin-AB]

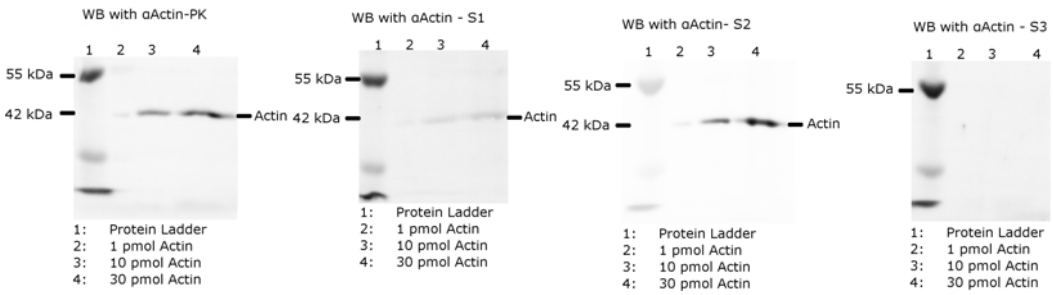


Figure 9: WB's scanned at 632 nm for detection of the αRabbit – AB-Cy5 used as 2nd AB. <u>From left to right:</u> Picture 1 shows signals for the detection of 1 pmol Actin in lane 1. Lanes 3 and 4 show signals for the higher amounts of actin. The conjugation product in picture 2 shows less intense signals but still visible for 1 pmol. Picture 3 shows more intense signals for 10 and 30 pmol actin in lanes 2 and 3 with the αActin–AB treated with Sulfo–SMCC but no primer is conjugated to. Picture 4 shows no signals for actin detection. The negative control used here as primary AB contains only the reduced primer P1.

In all samples were the αActin–AB was present, it was possible to detect the target actin. This means that the treatment of the AB with Sulfo-SMCC do not interfere with the target recognition of the AB.

A successful conjugation of primer P1 to the antibody could not be shown. For the next experiments higher concentrated antibody (αGST–AB (5 $\frac{mg}{ml}$)) and a primer (P2) with an additional Cy3 label between the nucleotides and the thiol group on the 5' end was used. This label could be detected directly on the heavy and the light chains of the AB, therefore the shifted chains can be discriminated from a possible cross conjugation of proteins.

3.1.3 Conjugation with αGST–Antibody

The used αGST–AB has a higher concentration. Therefore the concentration step can be circumvented. Validation of protein-DNA conjugation and protein processing was shown via denaturing SDS-PAGE. The conjugated primer P1 should lead to shift of about 12 kDa according to the molecular mass visible in a Coomassie stained gel. Primer P2 should lead to an even bigger shift (20 kDa), because of the additional Cy3-label.

S1: αGST–AB (40 µg) + P1 (600 pmol)

S2: αGST–AB (40 µg) + P2 (600 pmol)

S3: αGST–AB 40 µg

S4: P1 (600 pmol)

S5: P2 (600 pmol)

The conjugation was performed according to the protocol at page 18 and analyzed using a 15 % SDS-PAGE and a variation of the Blue Native Page (Wittig, Braun et al. 2006).

10 µl of each sample (S1 to S5), and 10 µl of the washing steps were load onto the gel and compared with 25 pmol αGST–AB.

Figure 10 to Figure 16 shows the successful conjugation process of the αGST-AB with primer P2. Figure 8 contains the conjugation products and the flowthroughs (FT) of the buffer exchange (W). The result of a second conjugation process is shown in Figure 11. This gel contain as additional control in lane 15 primer P2. In the following figures aliquots of the different washing steps during the conjugation process are shown.

Figure 10 contains the conjugation product of αGST-AB with the primer P1 (lane 3) and primer P2 (lane 4). In lane 3 the shifted bands for the heavy and light chains of the AB can be detected. The shift for each band is about 20 kDa. The molecular masses of light and heavy chain are about 24 and 50 kDa resp. (Figure 10, αGST - AB in lane 2 of the Coomassie stained gel). The signal of the Cy3 labelled primer on the light chain is at 44 kDa. The fluorescent signal at 70 kDa in Figure 10 is the shifted heavy chain. These signals are only visible for the conjugation product and do

not appear in the negative control of sample 2 (Figure 10, lane 6). The same signals of P2 in the lower part of the gels can be seen by comparing Figure 10, lane 4) with the negative control (lane 8).

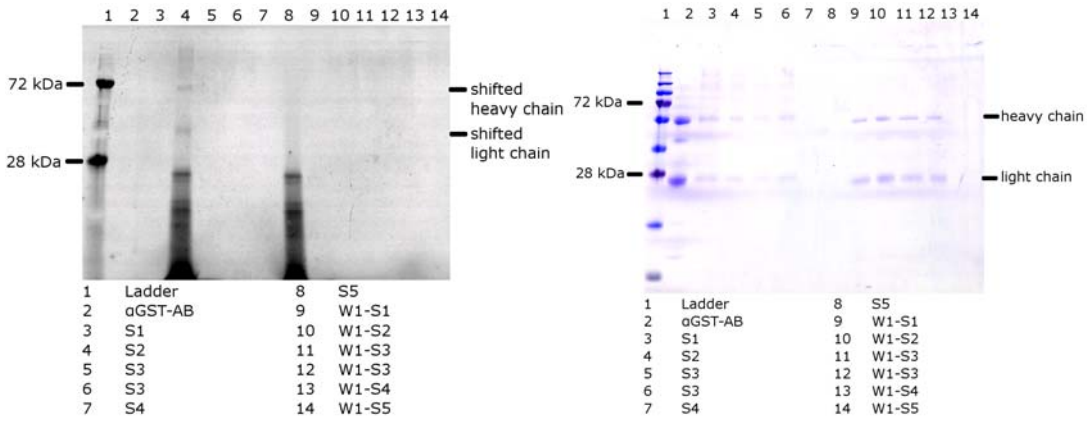


Figure 10: <u>left</u>: Fluorescence scanned SDS-PAGE of αGST-AB to DNA conjugation at λ_{em} 532 nm. The scan shows the Cy3-labelled primer P2. Lane 4 exhibits the conjugated primer to the heavy and the light chain of the αGST-AB. The lower signals are the unbound primer also shown in lane 8 (primer P2 alone as negative control). Lanes 9 to 14 contain the buffer exchange steps of αGST - AB prior to conjugation.

<u>right:</u> SDS - PAGE of αGST-AB to DNA conjugation. The Coomassie staining shows the heavy (50 kDa) and light (24 kDa) chains of the αGST-AB. The conjugation product of αGST-AB and the primers P1 (lane 3) and P2 (lane 4) is shown by the shifted heavy chains at 70 kDa (and higher bands due to multiple conjugation). These bands can not be seen in the negative controls of the conjugation (S3, lanes 5 and 6). The negative controls containing only the primer give no signal.

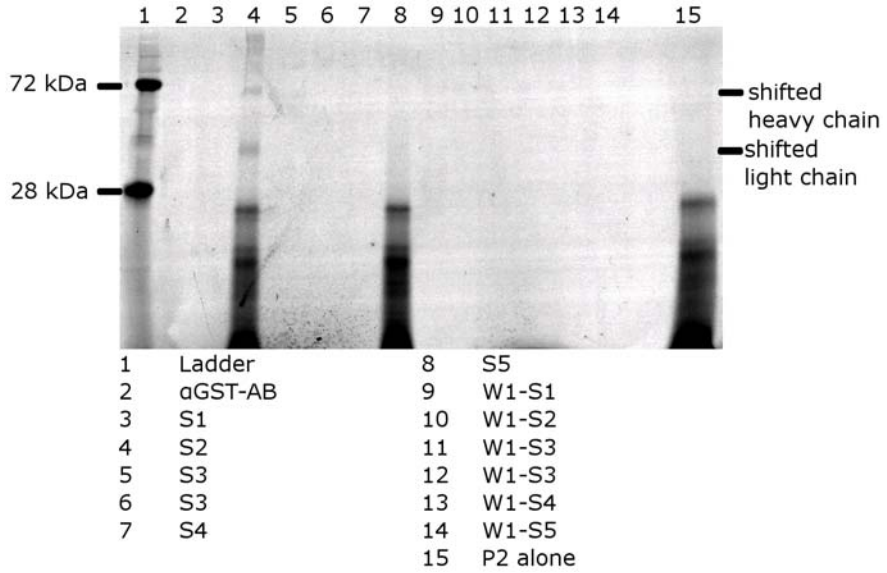


Figure 11: Fluorescence scan of SDS-PAGE with αGST-AB to DNA conjugation at λ_{em} 532 nm. The scan shows the Cy3-labelled primer P2. Lane 4 exhibits the conjugated primer to the heavy and the light chain of the αGST-AB. The lower signals are the unbound primer also shown in lane 8 (P2 alone as negative control) and 15 (P2 as delivered). Lanes 9 to 14 contain the buffer exchange steps of αGST-AB prior to conjugation.

Figure 9 shows an independent repetition of the conjugation process. Lanes 9 to 14 of Figure 10 contain 10 µl of the 50 µl FT from the washing steps. The signals in the

Coomassie stained gel in lanes 9 to 12 in Figure 10 are comparable to the ones of the conjugation product in lanes 3 to 6, indicating that a high amount of AB was lost at the washing steps.

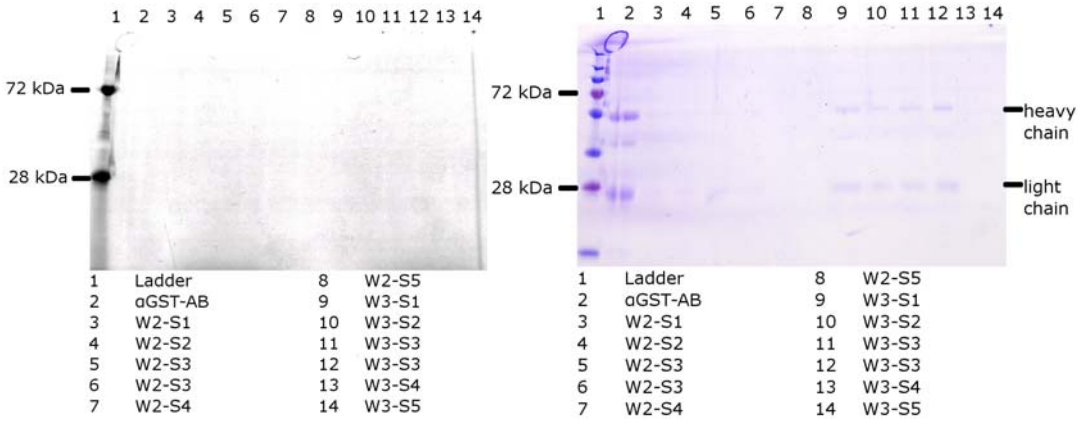


Figure 12: <u>left:</u> Fluorescence scanned SDS-PAGE of αGST-AB to DNA conjugation at λ_{em} 532 nm. Lanes 3 to 8 contain the FT of the 2nd washing step for buffer exchange steps of αGST-AB prior to conjugation. Lanes 9 to 14 contain 3rd washing step of these buffer exchange. <u>right:</u> Coomassie stained SDS-PAGE of αGST - AB to DNA conjugation.

In Figure 12 aliquots of the washing steps W (first buffer exchange) were loaded. One can see the same loss of αGST-AB in the W3 for all samples. As control 7 pmol αGST–AB in lane 2 were loaded.

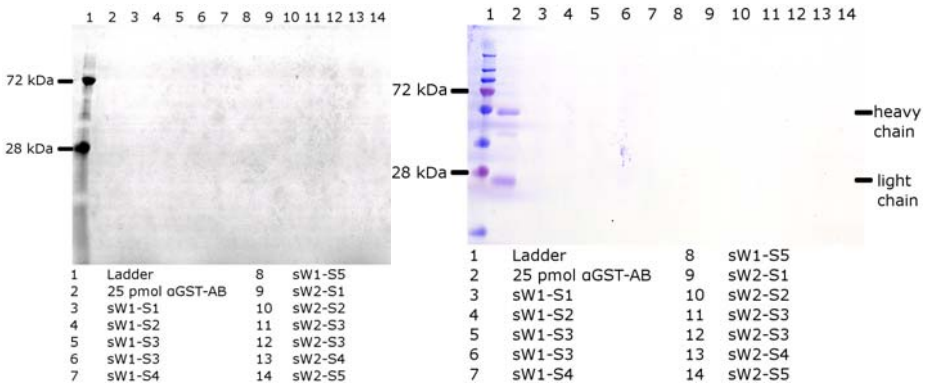


Figure 13: <u>left:</u> Fluorescence scanned SDS-PAGE of αGST-AB to DNA conjugation at λ_{em} 532 nm. Lanes 3 to 8 contain the FT's of the 1st washing step for separating unbound Sulfo–SMCC from αGST-AB. Lanes 9 to 10 contain the 2nd washing step for separating Sulfo–SMCC from αGST-AB.

<u>right:</u> Coomassie stained SDS-PAGE of αGST-AB to DNA conjugation.

The separation of unbound Sulfo–SMCC from AB (sW1 – sW3) is shown in Figure 13. Comparing lanes 3 to 14 with lanes 3 to 8 in Figure 14 shows no additional loss of αGST – AB or primer P2. It can be assumed that less than 10% of the starting material was lost before the conjugation to DNA can take place.

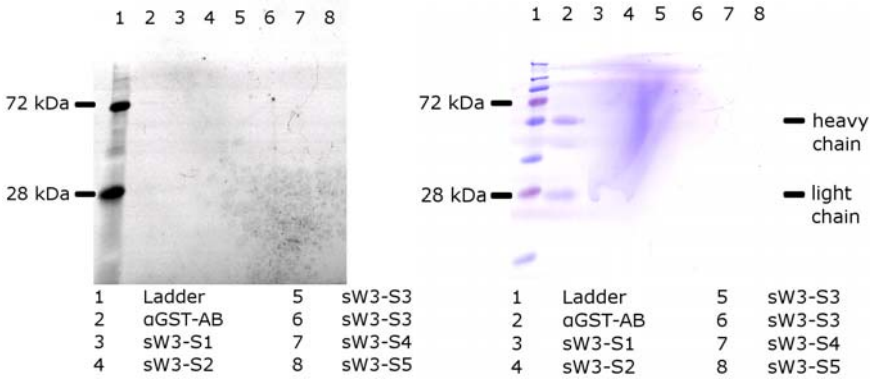


Figure 14: <u>left:</u> Fluorescence scanned SDS-PAGE of αGST-AB to DNA conjugation. Lanes 3 to 8 contain the FT's of the 3rd washing step for separating unbound Sulfo–SMCC from αGST-AB.

<u>right:</u> Coomassie stained SDS-PAGE of αGST-AB to DNA conjugation.

The separation of DTT from the reduced primers P1 and P2 was performed with G-50 Sephadex™ columns. 10 µl of each FT from the primer reduction were loaded together with 2 µl 100 % Glycerol on an 8 % PAA-Gel. 1 µl 100 µM P1 (100 pmol) as C1 and 0.25 µl 100 µM P2 (25 pmol) as C2 were loaded. DNA was detected by scanning at λ_{em} 532 nm Cy3 Signal and after staining with SYBRGEEN.

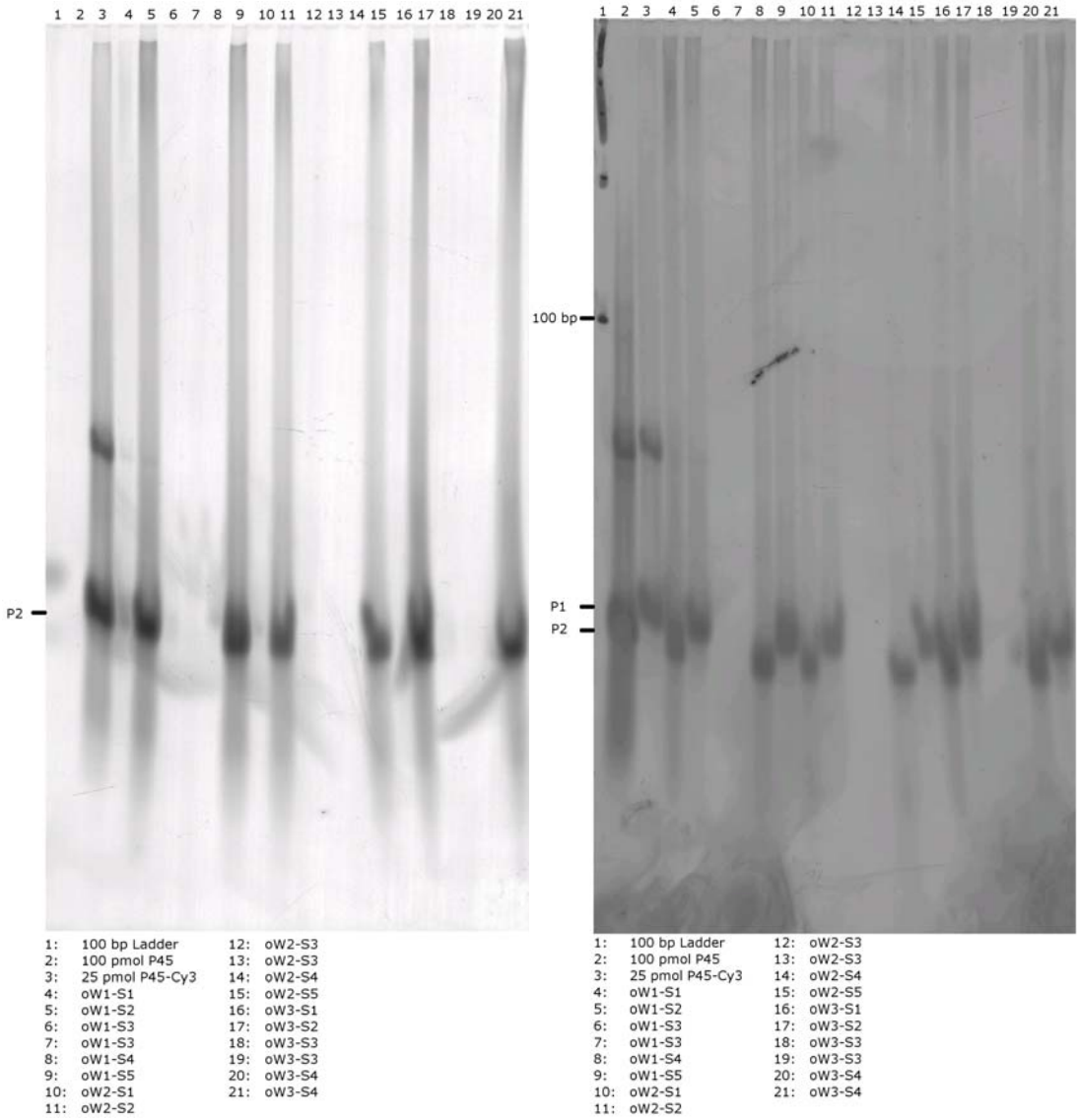


Figure 15: native PAGE of FT's from P1 and P2 for separation of primers from DTT. <u>left:</u> Fluorescence scan. <u>right:</u> Stained with SYBRGREEN

Figure 15 shows the washing steps to remove DTT from free primers after the reduction of the thiol groups. A difference between P1 and P2 can be seen, due to the additional Cy3 – tag at P2. Lane 2 contains 100 pmol of primer P1 and in lane 3 were 25 pmol of P3 loaded. Beside the monomers in lane 2 and lane 3 were also primer dimers visible. These dimers were removed during washing steps. With each washing step of samples S1, S2, S4 and S5 about 100 pmol primers are lost in the FT. The gel column showed a slight staining due to the Cy3-label of the P2 as well as the FT (oW1). After the second washing step (oW2) the smear of the lanes disappears. The loss of primers, which is documented in Figure 15 is due to the gel columns.

After successful conjugation of primers P1 and P2, the samples S1 – S5 were concentrated to achieve a higher concentrated sample for the following analysis. Table 18 summarizes the volumes applied to Amicon® ULTRA Centrifugal Filters for the αGST–AB–conjugate, the end volume and the flow through (FT).

Table 18: concentration of merged AB-DNA in Amicon®

	S1	S2	S3	S4	S5
Starting volume	150 µl	220 µl	450 µl	200 µl	200 µl
End Volume	75 µl	110 µl	220 µl	100 µl	100 µl
FT	75 µl	110 µl	230 µl	100 µl	100 µl

The FT of S2 (αGST-AB-P2) was clear but supernatant of S5 was stained with Cy3-dye. This means that almost every P2 must have bound to the protein. Figure 16 shows the αGST – AB conjugated with DNA before and after the concentration.

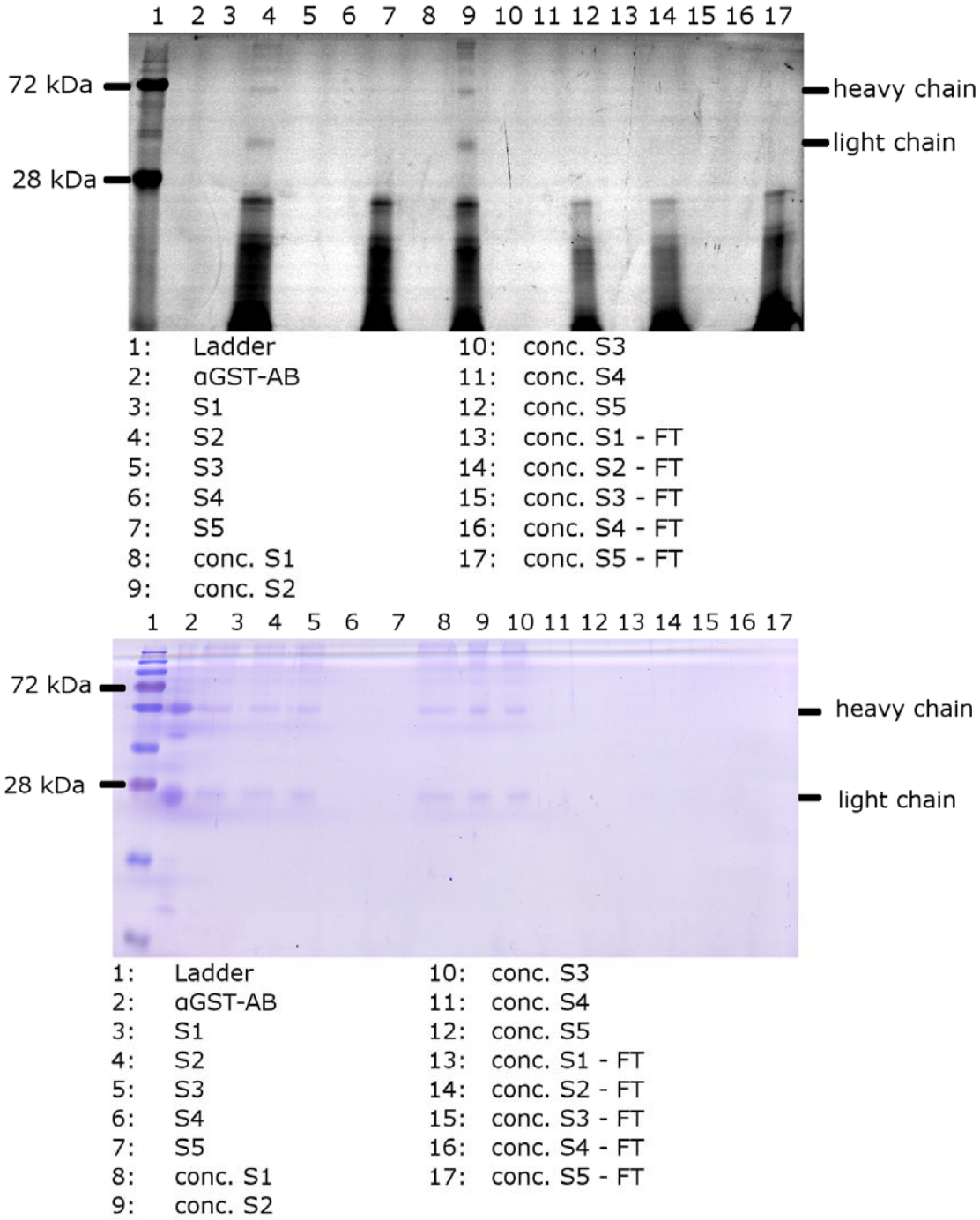


Figure 16: SDS-PAGE of αGST-AB. Lanes 2 to 6 contain the αGST-AB – DNA conjugation product and controls. Lanes 7 to 11 contain the αGST-AB – DNA conjugation products and controls after concentration with Amicon® 100 kDa MWCO (half the volume was applied to gel as for the not concentrated samples in lanes 2 to 6). The 10 µl FT volume of the concentration are in lanes 12 to 16. <u>upper part:</u> fluorescence scan at λ_{em} 532 nm <u>lower part:</u> Coomassie staining

10µl of sample before concentration and 5µl after concentration were loaded onto the gel. The intensity of the bands is comparable, indicating that nearly no protein was lost. From the gels above it can estimated that the concentration of the conjugated antibody is about 0.5 µM (~2.5 pmol, ~400 ng). Signals for the fluorescent primer P2 can be detected in the FT and in the concentrated sample S5. Even after

concentration of the samples the concentration of the conjugation product is not high enough to see the conjugated proteins in a Coomassie stained SDS-PAGEfor subsequent assays.

Silver Staining of PAGE

To validate the shift, silver staining of the denaturing gels were done. Silver staining has a lower detection limit compared with Coomassie. Figure 17 shows the silver stained PAGE for αGST–AB and the conjugation products. Lanes 3 (S1, αGST–AB-P1), 4 (2 αGST–AB-P2) and 5 (αGST–AB) show many bands between 25 kDa and 75 kDa. These bands are also visible in lane 2 (antibody not treated). The different size of the primers P1 (lane 3) and P2 (lane 4) can be seen in the lower parts of these lanes (compare to Figure 15). Additives added to stabilize the antibody hamper to detect the shift.

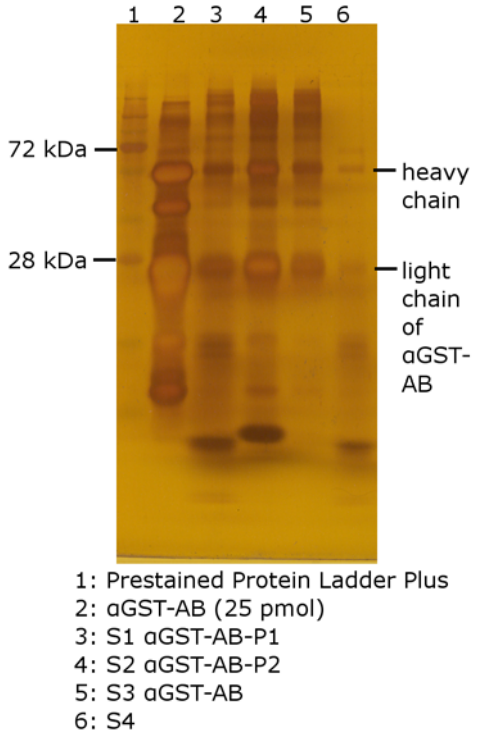


Figure 17: Silver stained SDS-PAGE. Lane 1 contains 25 pmol of the αGST-AB as delivered. In lanes 2 and 3 are the conjugation products of the αGST-AB and the primer P1 and P2 resp. Lane 4 contains the NC of S without any DNA. Lane 5 contains only the primer P1.

Silver staining was not convenient to discriminate the conjugation product of Protein / AB to DNA from the original protein, due to the high background signals of the additive materials stabilizing the AB. Blue Native PAGE was used as an alternative to

visualize the shift. Starting point is the native PAGE described by (Walker 1994) and as a second native gel system Blue native PAGE by (Wittig, Braun et al. 2006)

Native Page

The Walker gel system was used to check the migration behavior of the protein His-FilA and the αActin-antibody.

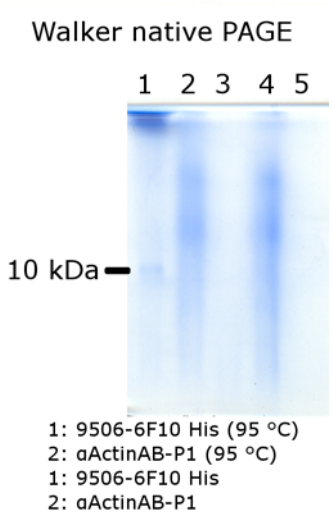


Figure 18: The Coomassie stained PAGE shows the 6 % Walker Native PAGE for Filamin A and αActin-AB, with and without denaturing step at 95°C.

Figure 18 shows the best result achieved with native PAGE. Samples were loaded without a denaturing step. The protein band appear still not as sharp and focused with a size of ~110 kDa for Filamin A and 144 kDa for the αActin-antibody. Therefore the blue native Gel system of Wittig *et al*. (Wittig, Braun et al. 2006) was tested..

Blue native PAGE was performed with several acrylamide concentrations of the gel. The difference of the Blue Native PAGE compared with native gels is the variation of two variables T, for percentage of the gel, and C, for the cross linking. T depends on the proportion of acrylamide to bisacrylamide in %, whereas C is depending only on the percentage of bisacrylamide in solution. Both values are independent of each other. A C value C = 3 for all gels and a percentage (T) of 12%, 10%, and 8% was chosen.

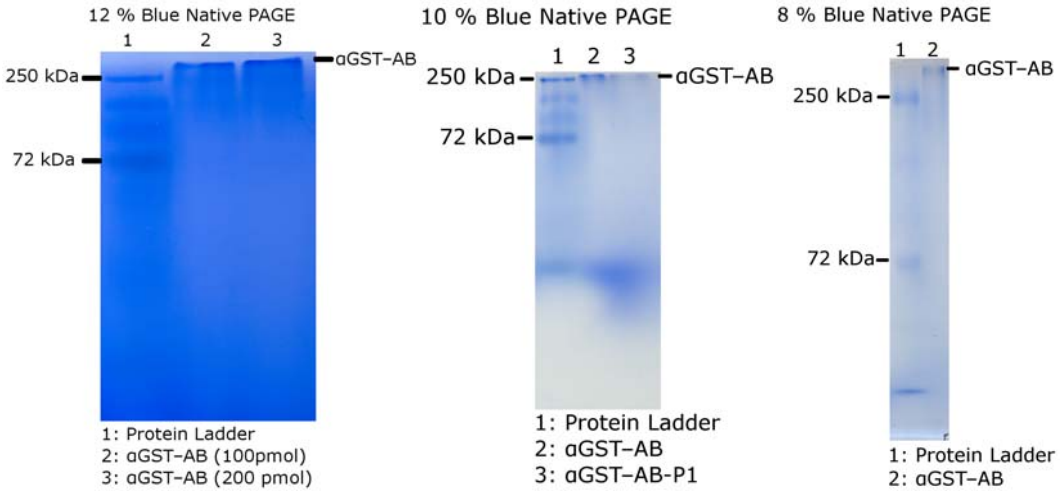


Figure 19: Coomassie stained Blue Native Page with different percentages to visualize a shift of the conjugated Protein / AB. All three different percentages of gels show no differences in the migration behaviour of αGST-AB.

Figure 19 shows results for the Blue Bative PAGE. The running buffer and the gel contain already Coomassie. Especially high percentage gels can be hardly destained. The 144 kDa αGST-AB can't enter the separation gel and tends to stick on the border of stacking to separation gel, regardless of percentage of blue native gel. Therefore no further attempt was made to check the conjugation.

The Cy3 labeled primer P2 was the matter of choice for demonstrating the conjugation to the AB. For the αGST–AB the conjugation of P2 could be successfully shown in lane 9 of Figure 16. For the conjugation of P1 to AB the proof was still missing, but due to the same treatment while conjugation process, one must assume similar results as for P2 conjugation. The successful conjugation was the basis for the next step, a functional test of the AB's with WB and the establishment of the RCA and the RCA on the DNA-AB conjugate.

3.2 Rolling circle amplification

The rolling circle amplification (RCA) was used for the linear amplification of a circular target DNA. The rolling circle product can be detected as high molecular DNA using PAGE or through the incorporation of labeled nucleotides (biotin, fluorescence labeled). The RCA was established in solution without AB and the optimized protocol adopted to perform the RCA on AB-DNA conjugates.

3.2.1 Generation of the circular template

Ligation of linear O2

The ligation of oligonucleotide O2 was done according to the protocol on page 26. Formation of the hairpin structure necessary for the ligation of the oligonucleotides, were tested at 7 different hybridization temperatures ranging from 20°C to 50 °C in 5°C steps. O2 was incubated at the respective temperature over night. After the ligation the samples were digested with Exonuclease VII to distinguish between linear and circularized DNA. From each ligation and digestion step 10 µl were analyzed on a 3% agarose gel and compared with 100 pmol linear O2. All samples were mixed with 2 µl 6 x DNA loading dye.

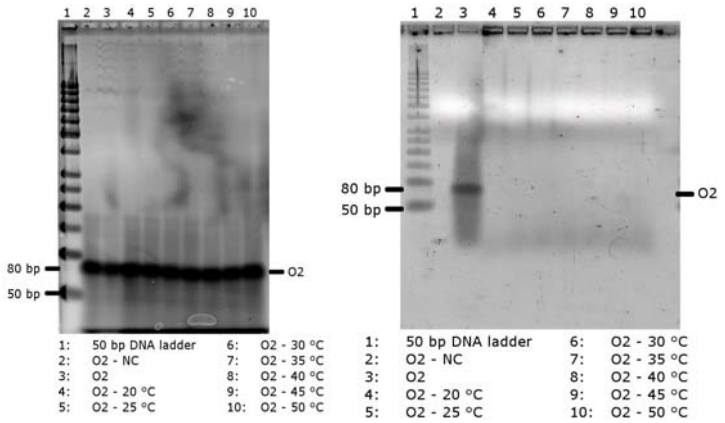


Figure 20: left: SYBRGreen stained 3 % agarosegel gel. 10 µl of each ligation assay were load to the gel with 2 µl 6 x DNA loading buffer. The gel was scanned at the Fluorescence scanner at λ_{em}473 nm wavelength. right: samples after Exonuclease VII digest O2 after ligation. Lane 2 contains 100 pmol of linear O2 for comparison. Lane 1 shows a sample without T4-ligase, containing the negative control of the ligation assay without T4-ligase. Lane 2 contains 100 pmol of linear O2. Lanes 4 to 10 contain the ligation products of O2.

Figure 20 shows the influence of the hybridization temperature on the circularization. Lane 2 contains a sample without T4 Ligase as negative control. The samples were completely degraded by Exonuclease VII. None of the hybridization assays for O2

led to stable circular product. O1 that can form a slightly different hairpin structure was tested with the same temperatures.

Ligation of linear template O1

Phosphorylation, ligation and Exonuclease VII restriction steps for circularization of oligonucleotide O1 is shown in Figure 21. The predicted melting temperature for oligonucleotide O1 was 41 °C. Hairpin formation was tested at 7 different hybridization temperatures ranging from 20°C to 50 °C in 5°C steps. The linear O1 migrates slower through a 8 % PAA–gel as the circular form (compare lanes 2 to 10). The upper bands in lanes 6 and 7 were multiple ligation products (concatemers) of oligonucleotide O1. They were not resistant against Exonuclease VII treatment (lanes 9 and 10), This means that they were still linear. As a rough estimation about 90% of oligonucleotide O1 was circularized.

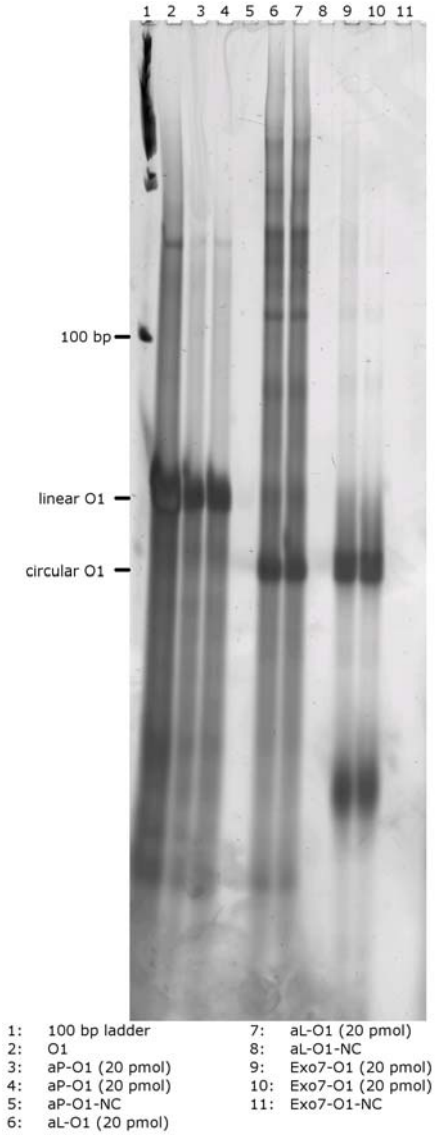


Figure 21: SYBRGreen stained PAGE of the generation process for the circular RCA – template from the linear form (lane1). Lanes 2 and 3 containing 20 pmol O1 after of the phosphorylation, lanes 6 and 7 after ligation and lanes 9 and 10 after ed O1. The circular O1 is visible in the lower band in lanes 6 and 7 and remain stable after Exonuclease VII treatment (lanes 8 and 9).

3.2.2 RCA on generated template O1

RCA of O1 with P1 / P2

The circularized and Exonuclease VII (Exo7) treated O1 was used as RCA template. 50 pmol P1 and P2 were hybridized to 25 pmol Exo7 treated circular O1 (Exo7-c-O1) and incubated with Phi29 polymerase. The RCA-Product (RCP) was high molecular and appeared in the upper part of the lanes after RCA. An example of the RCA is shown in Figure 22. A 100 bp ladder was loaded as size standard and the linear oligonucleotides P1 (100 pmol), P2 (12,5 pmol) and linear O1 (50 pmol). 5 µl of the assays before and after RCA were mixed with 2 µl 100 % Glycerol and loaded onto an 8% PAGE. The gel is scanned at λ_{em} at 473 nm and λ_{em} 532 nm to visualize the Cy3 labelled primer P2 and after SYBRGreen staining to detect the amplified DNA.

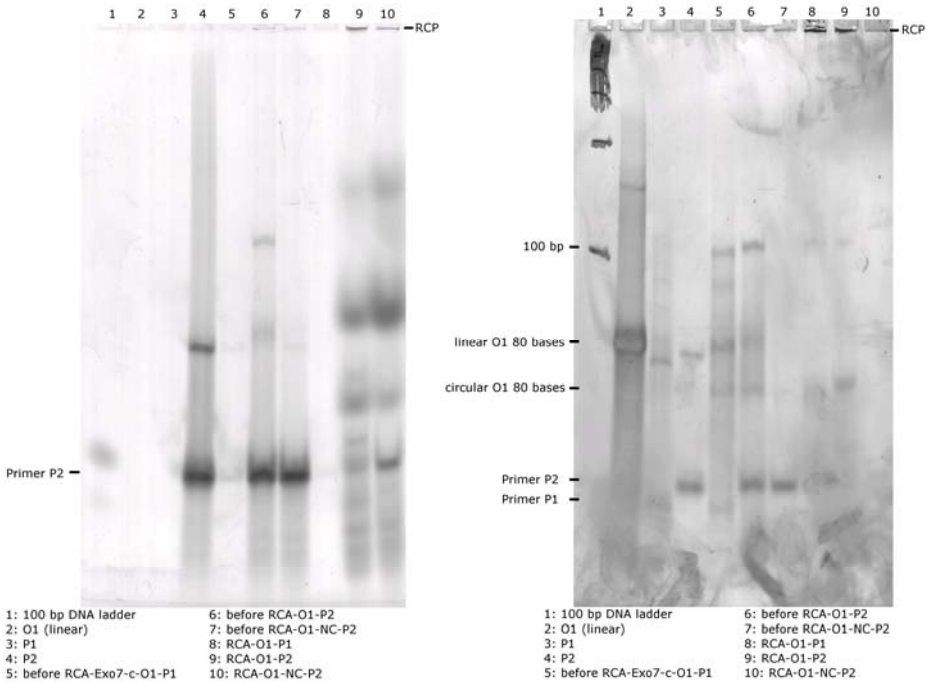


Figure 22: <u>left:</u> Fluorescence scan of the PAGE for validation of the RCA at λ_{em} 532 nm. Lane lanes 9 and 10 contains the high molecular RCP of the circular O1 and the Cy-3 labelled primer P2 as high molecular material in the upper parts can be detected. The RCP is not present in lane 6 (before RCA). Primer P2 is shown in lane 4. <u>right:</u> PAGE after SYBRGreen staining of the same PAGE. The RCP as high molecular DNA can be seen in lanes 8 (circular O1 and P1) and 12 (circular O1 and P2).

The RCP appears as “smear” in the upper part of the lanes 8 and 9 containing the RCA assay. In lane 9 the Cy3 label of primer P2 can be detected after RCA in the pockets of the gel. P1 and P2 were able to hybridize to the template DNA. The RCP for both primers was of the same intensity on the gel after SYBRGreen staining. The RCA with the circularized template O1 and the primer P1 and P2 was successfully

established. The next step is the RCA with the primer conjugated to the αGST-AB.

3.3 RCA on generated template and DNA-Antibody conjugate

The assay should show if primers P1 and P2 after conjugation to an AB can still serve as starting site for the RCA. The hybridization of the circular template to the DAN-AB product was done prior to the RCA. To avoiding the denaturation of the antibody the incubation at 75°C to inactivate the ligase was omitted. The hybridization of O1 to the conjugated primer was done at 37°C. For comparing the RCP without AB the RCP samples shown in figure 20 were load as controls. Additional controls were 50 pmol of linear O1, 12.5 pmol P2 and 5 µl of RCA assays.

Table 19: Content of samples for RCA on αGST–AB-DNA using the circular O1 after Exonuclease VII treatment as template DNA.

sample	αGST–AB-P1	αGST–AB-P2	αGST–AB	O1	P1	P2
RCA-O1-P1	-	-	-	25 pmol	50 pmol	-
RCA -O1-P2	-	-	-	25 pmol	-	50 pmol
RCA -O1-S1	3.75 pmol	-	-	12.5 pmol	-	-
RCA -O1-NC-S1	3.75 pmol	-	-	-	-	-
RCA -O1-S2	-	3.75 pmol	-	12.5 pmol	-	-
RCA -O1-NC-S2	-	3.75 pmol	-	-	-	-
RCA -O1-S3	-	-	3.75	12.5 pmol	-	-
RCA -O1-NC-S3	-	-	3.75	-	-	-
RCA -O1-S4	-	-	-	12.5 pmol	-	-
RCA -O1-NC-S4	-	-	-	-	-	-
RCA -O1-S5	-	-	-	12.5 pmol	-	-
RCA -O1-NC-S5	-	-	-	-	-	-

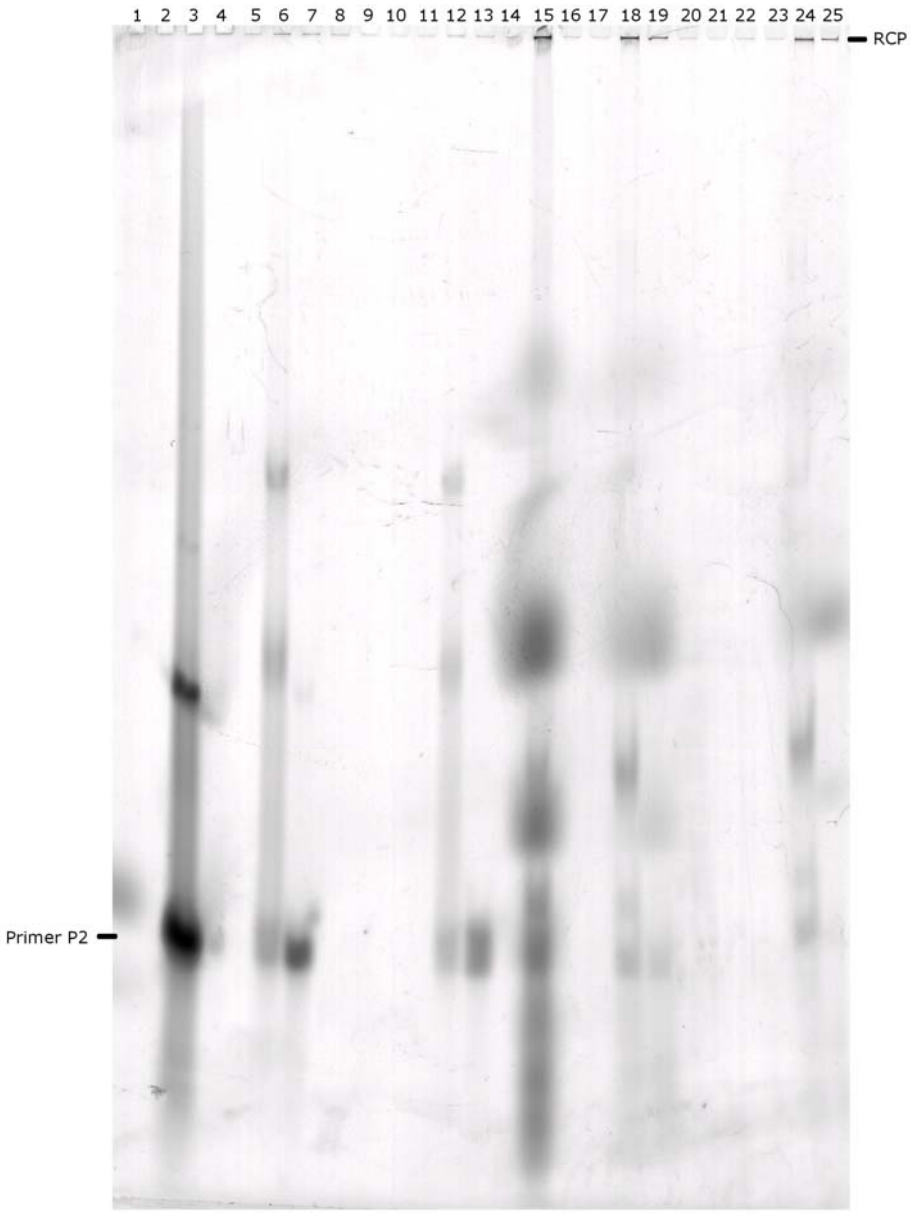


Figure 23: Fluorescence scan of PAGE at 532 nm. The RCP on the αGST-AB could successfully be established (compare lanes 6 and 18)

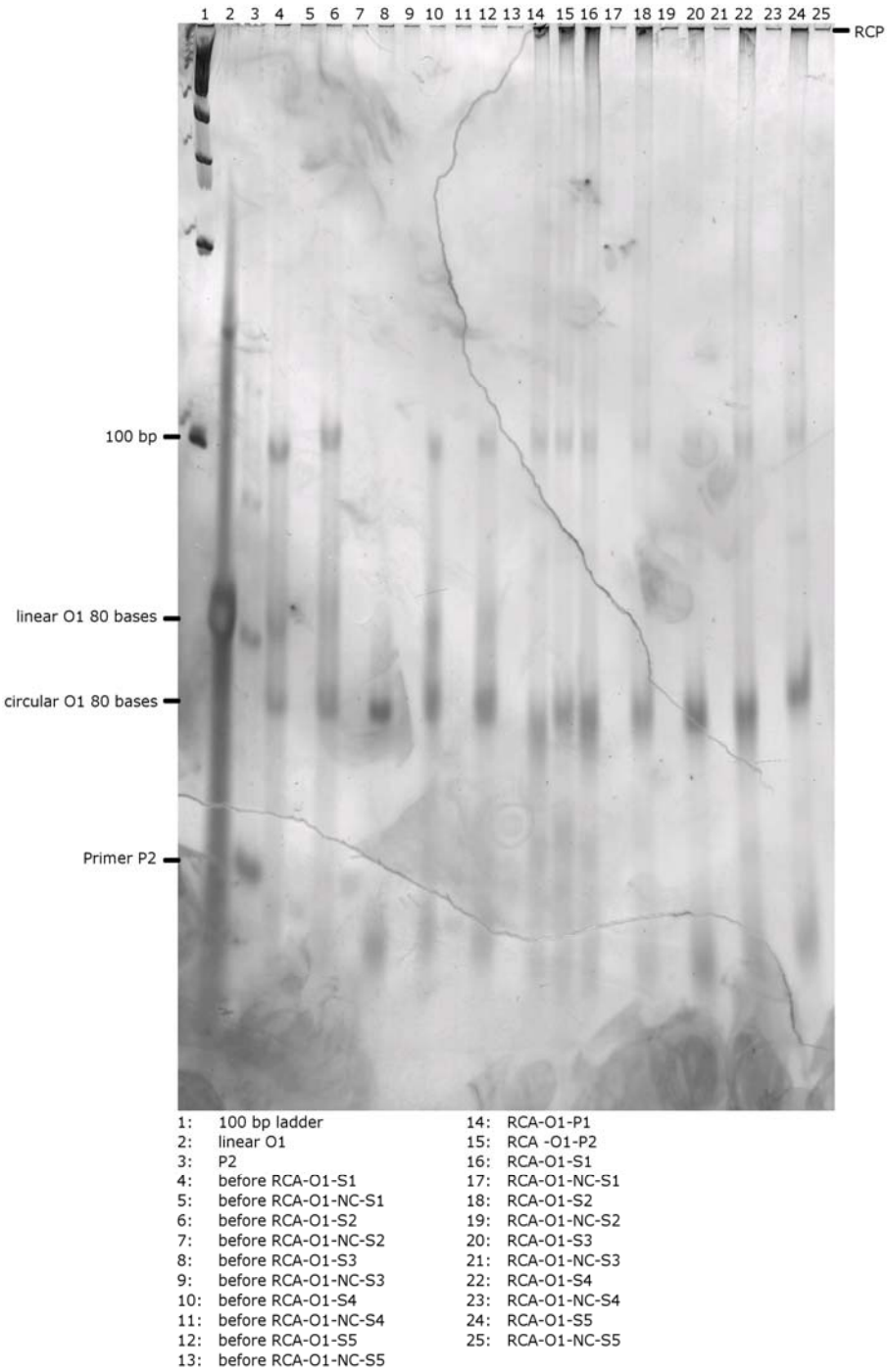


Figure 24: SYBRGreen stained PAGE λ_{em} scanned at 473 nm. The RCP is in the upper part of lanes 14 (RCP with P1), 15 (RCP with P2), 16 (RCP with P1 on αGST-AB), 18 (RCP with P2 on αGST-AB). The RCP in lanes 22 (P1 only) and 24 (P2 only) is due to the unbound P1 / P2. The negative controls in lanes 17, 19, 21, 23 and 25 containing no RCP (no template DNA).

Figure 23 and Figure 24 show the RCA products with the DNA-AB conjugate. The PAGE show the same samples before and after RCA. The signal intensity of P2 alone (Figure 23, lane 15) and bound to αGST-AB (Figure 23, lane 18) was identical. The conjugation of a primer to an AB did not inhibit the RCA. For the P2-AB conjugate the amplified DNA was already detectable without SYBRgreen staining

(Figure 21). The RCP products with both primers can be seen in the SYBRGreen stained PAGE (Figure 24). Lanes 14 and 15 contain the RCP of P1 and P2 resp. and were both of the same intensity. Also the P1 conjugated to αGST-AB leads to high molecular DNA in the upper part of lane 16. This means although the conjugation of primer P1 could not be confirmed the primer P1 was successfully conjugated. The intensity of conjugated primer was diminished compared to the intensity of the free primer lane 18 (P2 conjugated to αGST-AB). The negative controls of all assays didn't show any detectable RCP. Amplification without primer was not possible.

The RCA using DNA conjugated antibodies was successfully established for both primers.

3.3.1 RCA of template O1 with P1 / P2 on Antibody with modified dUTPs

For the final test of the conjugated DNA–AB, the RCA was performed on a membrane and the RCP detected by incorporation of:

i) Cy-3 dUTP resulting in a fluorescent RCP

ii) biotin-dUTP combined with subsequent interaction with Cy3-Streptavidin

In both cases modified nucleotides were incorporated during the amplification step. Cy3-dUTP can be detected without any additional step. Whereas after incorporation of biotin-dUTP an additional incubation step with Cy3-Streptavidin was necessary. Basis for these two tests were first the functionality of the αGST–AB and second the successful RCA using modified dUTP's. First the RCA was tested with both modified dUTPs and primers P1 and P2 conjugated to the AB in solution.

Table 20: Content of samples for RCA on αGST–AB-DNA using the circular O1 after Exonuclease VII treatment as template DNA and modified dUTP's

sample	αGST–AB-P1	αGST–AB-P2	αGST–AB	O1	P1
RCA-O1-P1	-	-	-	25 pmol	50 pmol
RCA-O1-P1 Cy3-dUTP	-	-	-	25 pmol	50 pmol
RCA-O1-P1 biotin-dUTP	-	-	-	25 pmol	50 pmol
RCA-O1-NC-P1	-	-	-	-	50 pmol
RCA-O1-S1 Cy3-dUTP	3.75 pmol	-	-	25 pmol	-
RCA-O1-S2 Cy3-dUTP	-	3.75 pmol	-	25 pmol	-
RCA-O1-S3 Cy3-dUTP	-	-	3.75 pmol	25 pmol	-

In all lanes containing the complete RCA mix (DNA-AB + template) and Cy3-dUTP a high molecular RCP was detected. After SYBRGreen staining an identical product could be detected also in lanes with biotin-dUTP. The signals are as intense as for the RCA using unmodified dTTPs (compare Figure 23 right lane 11(dTTP), lane 12 (Cy3-dUTP) and lane 13 (biotin-dUTP). The RCP with Cy3-dUTPs of αGST-AB-P1 (lane 15) was less intense in the Cy3 scan as the one for the αGST-AB-P2 (lane 16). This could be due to additional Cy3 label on the conjugated primer P2. The results indicate a successful RCA for both DNA-antibody conjugates and the successful incorporation of modified nucleotides. The RCA without DNA-AB has a higher efficiency.

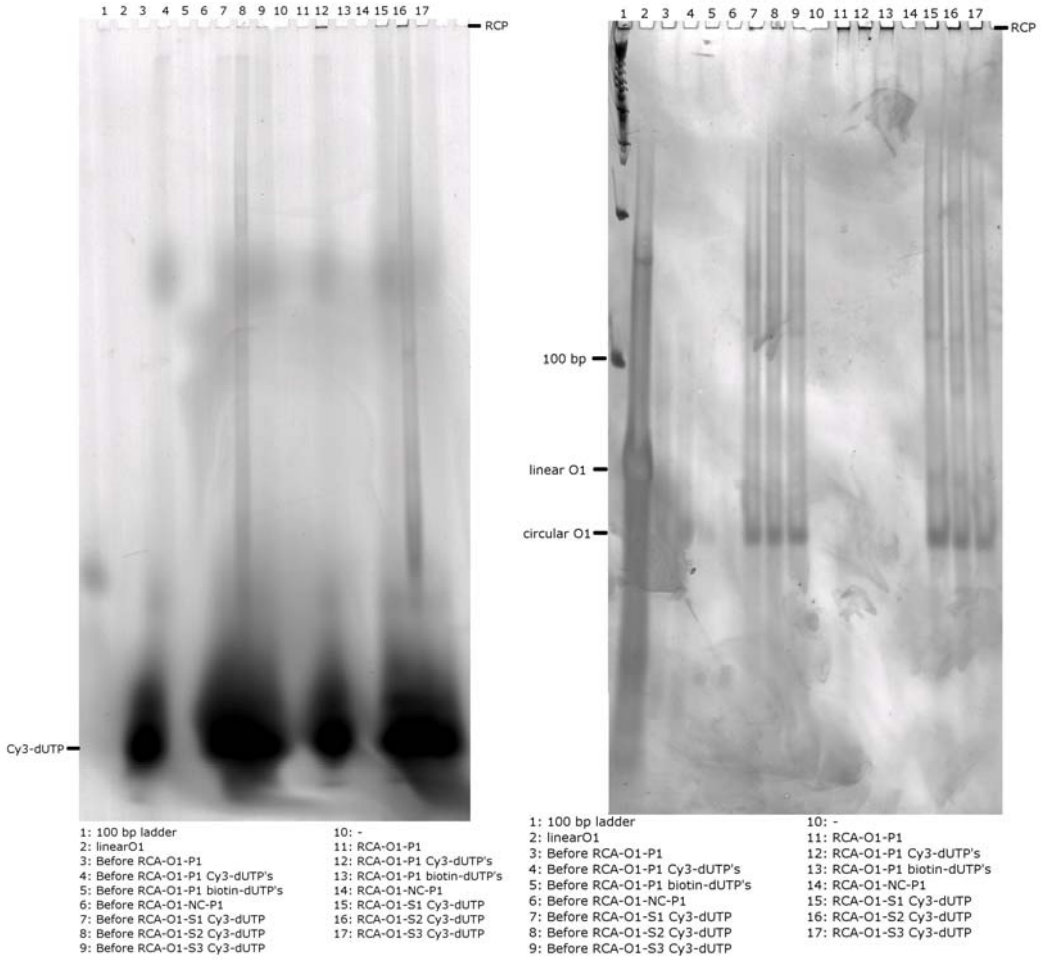


Figure 25: <u>left:</u> Fluorescence scan of PAGE containing RCP of assay with modified dUTPs at λ_{em} 532 nm. Lane 12 contains the RCP using Cy3 labelled dUTP and P2. It is as intense as the RCP signal in lane 16 using P2 conjugated to αGST-AB and the Cy3 labelled dUTP. Lane 15 shows a less intense RCP signal using αGST-AB with conjugated P1 and Cy3-labelled dUTP. <u>right:</u> Scan of SYBRGreen stained PAGE λ_{em} 473 nm containing the RCP's of Cy-3 dUTP (lane 12), biotin dUTP (lane 13) and dTTP (lane 11) with P1 hybridized to circular template O1. All signals are of same intensity. Lane 15 (αGST-AB-P1, S1), 16 (αGST-AB-P2, S2) and 17 (αGST-AB without primer (S3)) showing the RCA using Cy3 labelled dUTP on AB. RCP signals for S1 and S2 are of the same intensity but not as intense as the RCA without the AB.

3.3.2 Functionality of DNA-AB conjugate

Beside the possibility to use a DNA-AB conjugate as template for a RCA the functionality of the modified was of interest. Therefore a dilution series of GST was blotted and incubated with the DNA-AB conjugate, unmodified AB and αDonkey–AB–Cy5 as secondary AB. The WB's were incubated with the following antibody conjugates:

S1: αGST-AB with primer P1 S3: αGST-AB without primer

S2: αGST-AB with primer P2 S4: only primer P1

S5: only primer P2

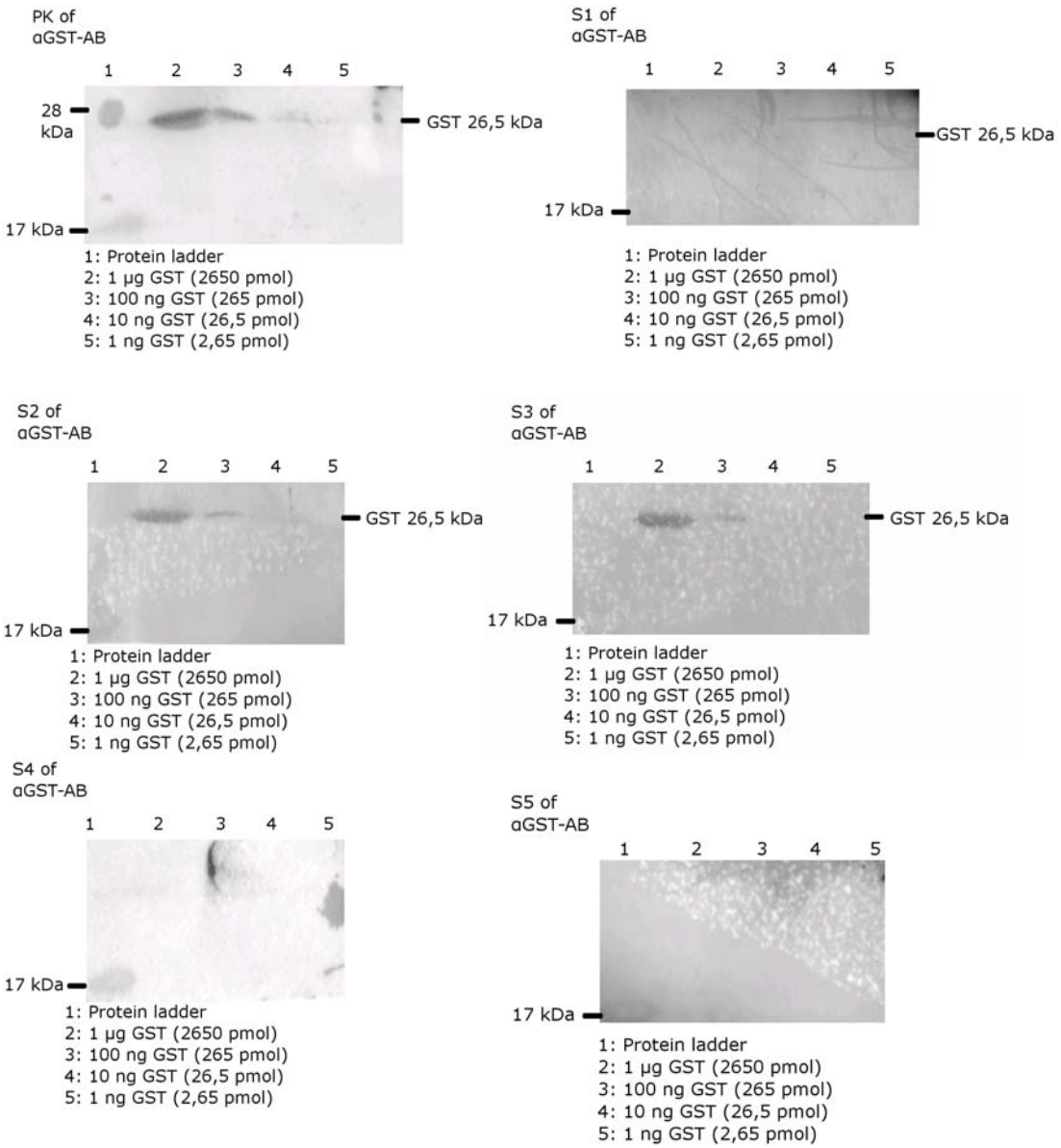


Figure 26: Fluorescence scans of WB at λ_{em} 635 nm. The WB of αGST-AB (PK) shows a signal for 1 µg, 100 ng and 10 ng GST. Conjugation products of αGST-AB (S2 + S3) show comparable signals. Only αGST-AB–P1 (S1) gives no signal. S4 and S5 are negative controls containing no αGST-AB.

Figure 26 show that the modified AB's were still able to recognize the target. GST was immobilized on nitrocellulose membrane in a dilution series ranging from 1000 ng to 1 ng GST. From the conjugation samples S1, S2 and S3 approx. 4 pmol modified AB were used. For samples S2 and S3 the signal intensities were comparable to each other and only slightly weaker than for the unmodified AB. S1 (αGST-AB with primer P1) gave no signal. In a second experiment also the sample S1 (conjugation of primer P1) gave a signal at λ_{em} 649 nm (Fig. 24). The detection limit is between 1 µg and 100ng GST. The results show that the conjugation did not interfere with the antigen specificity

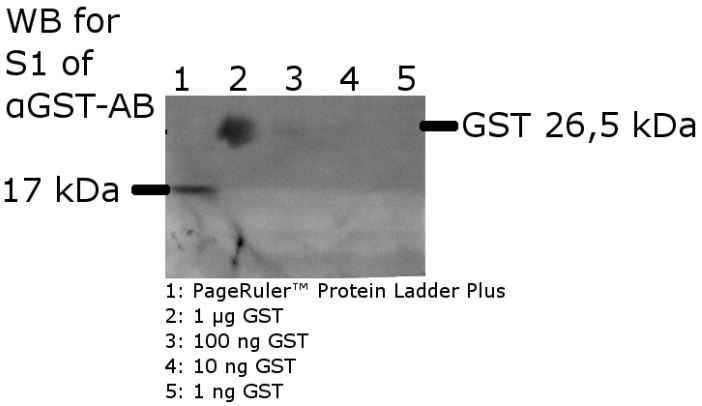


Figure 27: WB scanned at λ_{em} 635 nm for detection of αDonkey–AB–Cy5 against αGST-AB-P1.

The experiment clearly shows that the antibodies after conjugation were still functional. The DNA can serve as starting site for RCA amplification in solution (see 3.3.1). The final test was the detection of the antigen GST directly on a membrane by incorporation of modified nucleotides during the RCA.

3.3.3 Membrane bound RCA on the DNA – antibody conjugate

A GST dilution series was blotted and incubated with DNA-AB-P1 and DNA-AB-P2 conjugate. The subsequent RCA was performed directly on the membrane using a small Petri dish for keeping a constant humidity during the RCA.

The pre-hybridized samples (DNA-AB with circularized O1) were prepared as described on page 32 and the GST-WB was incubated as described on page 36.

Membrane bound RCA on the DNA – AB conjugate using Cy3-dUTPs

The assay should give a fluorescent RCP The RCA was performed with the pre-hybridized circular template O1. This pre-hybridized product was used to recognize membrane bound GST for all samples of conjugation.

S1: αGST-AB with primer P1

S2: αGST-AB with primer P2

S3: αGST-AB without primer

S4: only primer P1

S5: only primer P2

For all samples 12.5 pmol modified αGST-AB was used as 1st AB and compared with 33 pmol unmodified αGST-AB as positive control. The RCA was done with 50 µl RCA-solution for 3 h at 30 °C. The membranes were washed and scanned at λ_{em} 532 nm.

For αGST-AB-P1 (S1) and αGST-AB-P2 (S2) a band corresponding to 1 µg GST was detected. Surprisingly also in the positive control (PK of αGST-AB) a signal was detectable. It cannot be ruled out that Cy3-dUTP stick to the unmodified antibody. The blots for αGST-AB-P2 and αGST-AB-P2 lead to the conclusion that Cy3-dUTP was incorporated in the RCP on the DNA-AB conjugated, leading to a fluorescence signal for both assays. The αGST-AB without primer (S3) did not lead to any signal. Compared with a classical sandwich approach (see above) the RCA assay was less sensitive. Taking into account that only a minor part of the antibody was conjugate with a DNA fragment and only those antibodies that have such a label can serve as template for RCA a higher sensitivity can be reached after optimizing the labelling procedure.

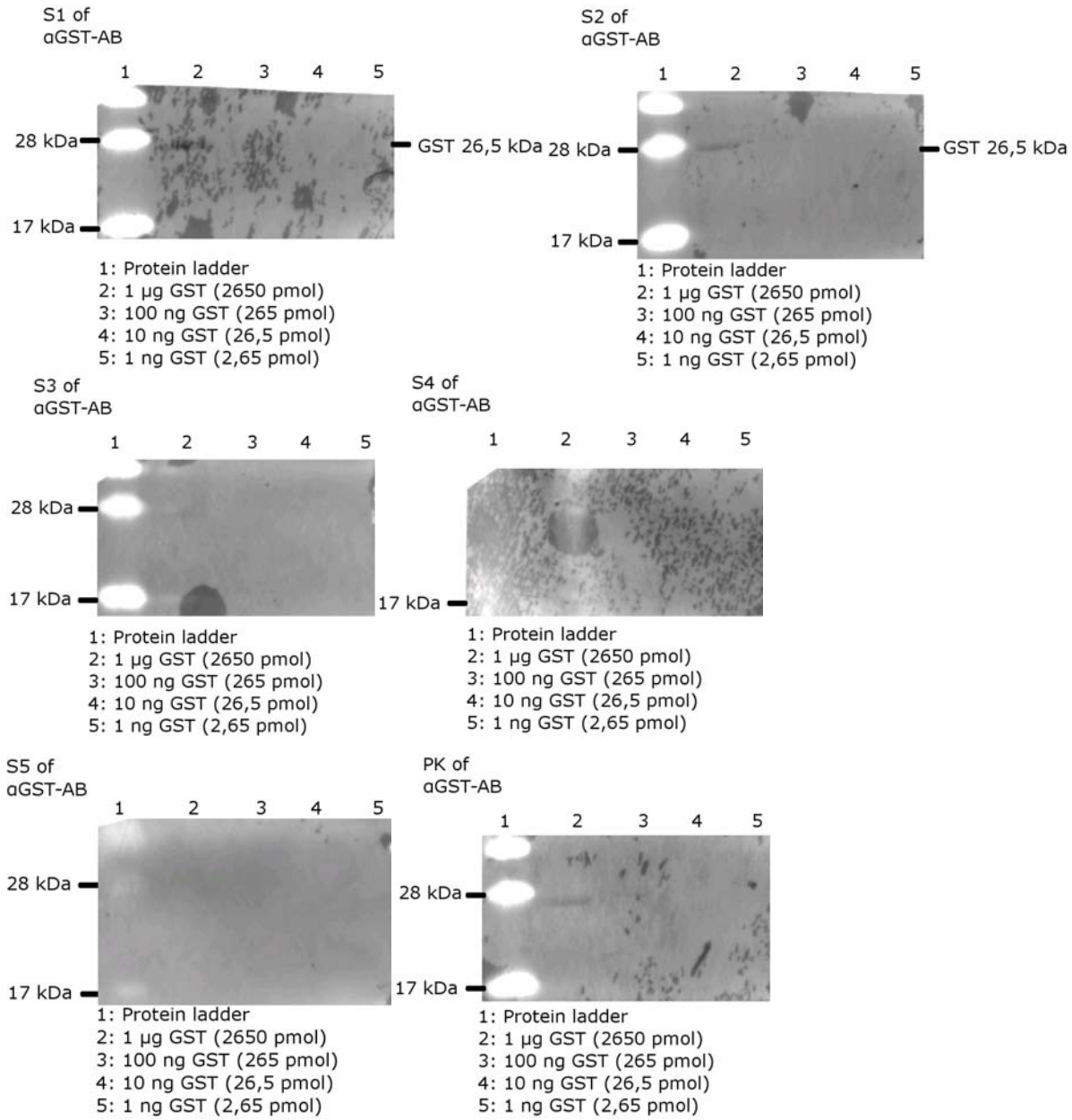
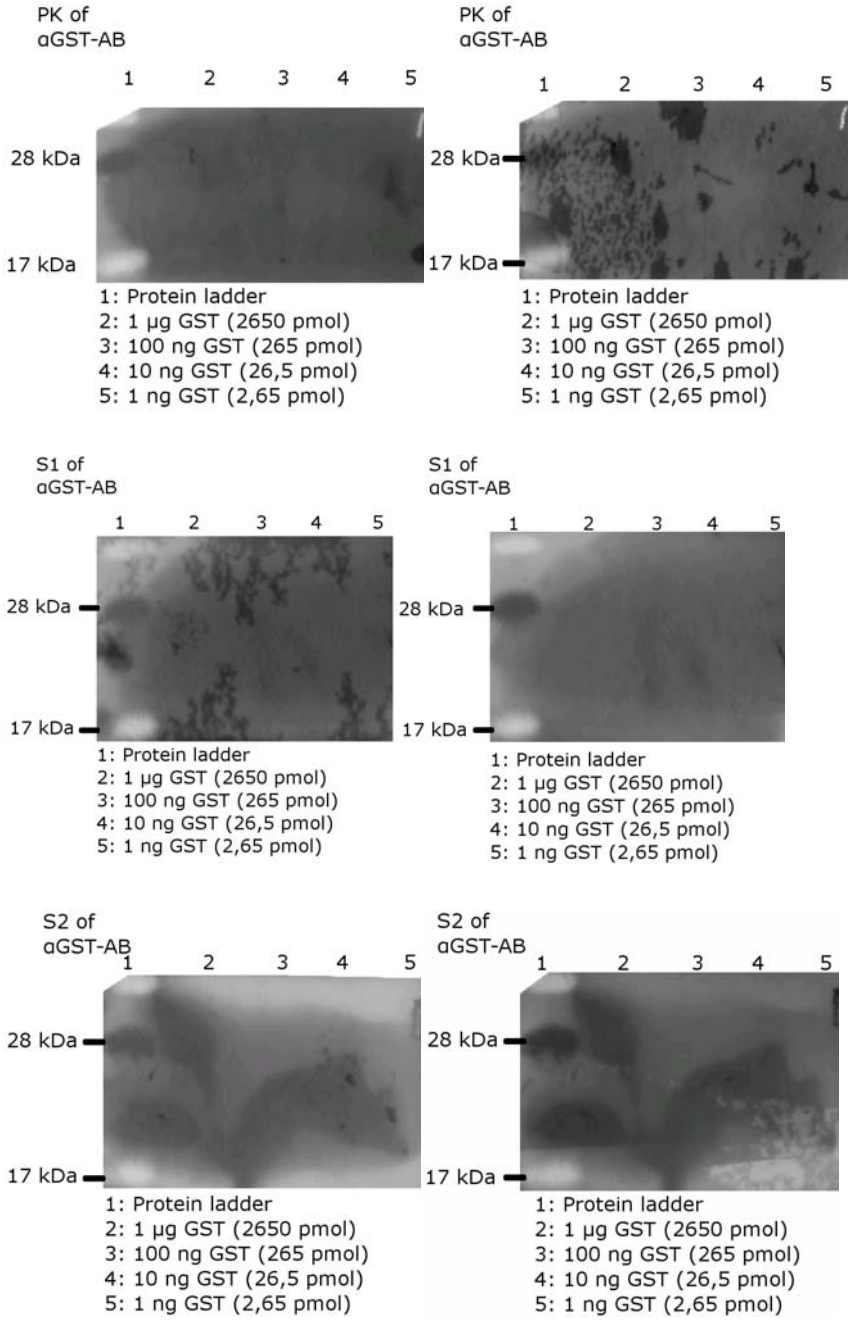


Figure 28: Fluorescence scan of WB at λ_{em} 532 nm for detection of Cy3-labeled dUTPs in RCP. RCA on WB using 12.5 pmol DNA-AB conjugate and 33 pmol αGST-AB as PK. S1 contains the P1 conjugation product, S2 the P2 conjugation product, both leading to a signals at 1 ng (45 pmol) GST. S3 contains no primer on αGST-AB and shows no signal. Also both negative controls (S4 and S5) don't show a signal.

In a second assay the incorporation of biotin labelled dUTPs as substrate for the RCA and hybridization with Cy-3 labelled Streptavidin was tested.

Membrane bound RCA on the DNA – AB conjugate using biotin-dUTPs

Biotin labelled dUTP used in this assay can interact with Cy3 – labelled Streptavidin to visualize the RCP on the DNA-AB conjugate. The DNA-AB conjugate was hybridized with circular O1 template DNA followed by incubation on WB. The RCA on membrane was done directly on the membrane as described on page 36. For all samples 12.5 pmol modified αGST-AB was used and compared with 33 pmol unmodified αGST-AB as positive control. The RCA was done with 50 µl RCA-solution containing biotin-dUTPs for 3 h at 30 °C. After washing the membranes were scanned at λ_{EM} 532 nm to detect Cy3-Strepavidin.



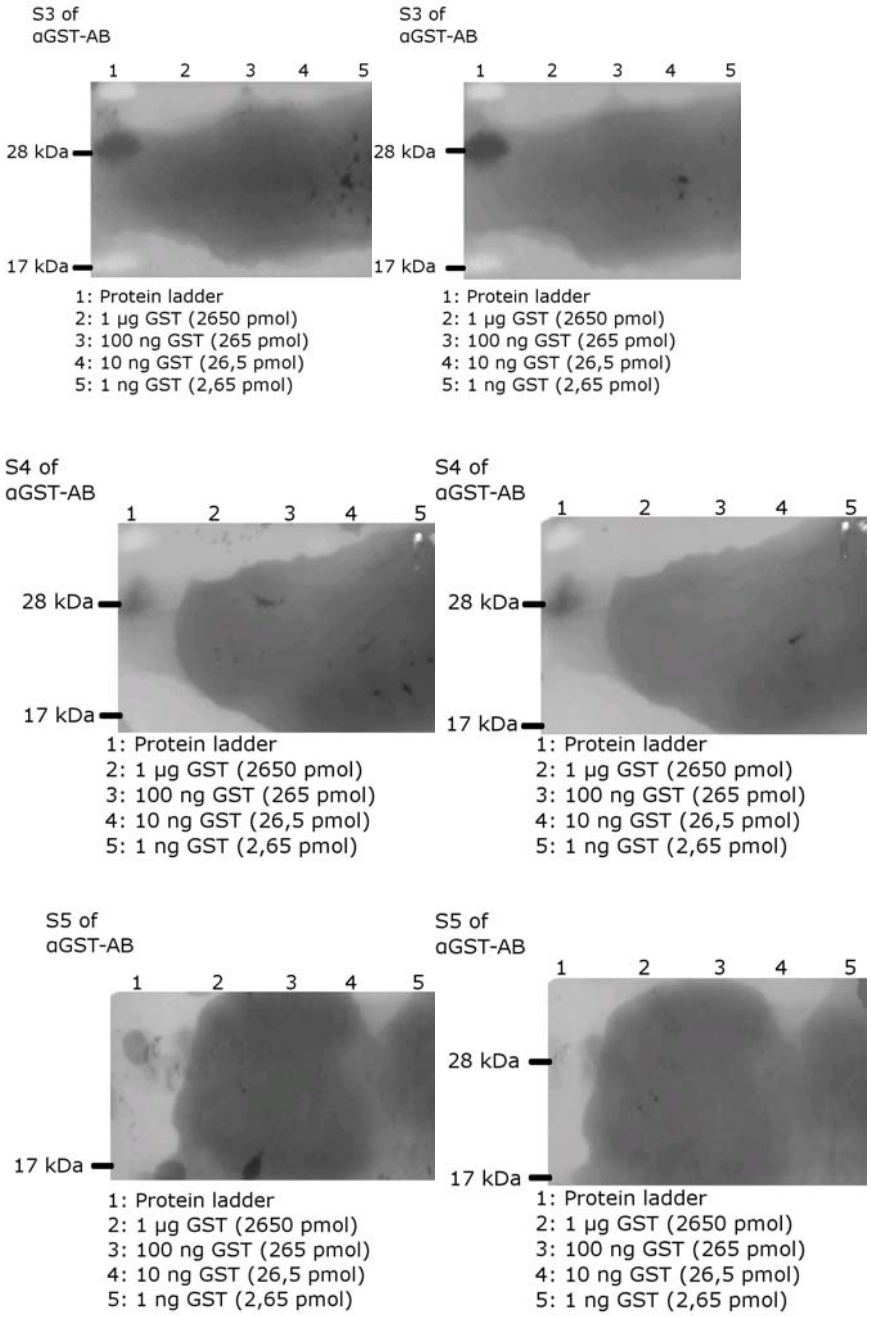


Figure 29: The WB's scanned at λ_{em} 532 nm before (left pictures) and after incubation with Cy3-Streptavidin. No signal can be detected.

Figure 29 shows the WB's with an immobilised dilution series of GST after incubation with DNA-AB conjugation products. The left pictures are taken before the incubation with Cy3-Streptavidin can interact with the RCP (left) and after with Cy3-Streptavidin (right) incubation. Unfortunately no signals could be detected.

The RCA could be established with the single stranded circularized template O1 and the thiol modified primers P1 and P2. The additional Cy3-label of P2 did not interfere with RCA efficiency. The RCA protocol based on Köster *et al*. (submitted) could be

expanded to RCA on DNA-AB conjugation products. A RCA with template O1 and modified dUTPs was possible. The RCA with modified dUTPs was also established for the conjugated antibodies in the presence of modified nucleotides. The functionality of the conjugation product was validated, without significant difference to unmodified antibody. The combination of RCA with modified dUTPs on the DNA-AB product, bound to membrane need to be optimized.

4 Discussion

The first part of the discussion deals with the conjugation process and the different approaches for visualizing the DNA-protein/antibody conjugation. In the second part the focus is on the rolling circle amplification, especially the optimization of the template DNA preparation and the transfer of the protocol to DNA-antibody conjugates in solution and on membranes.

4.1 DNA conjugation

The conjugation of DNA to a protein or an AB was analyzed by detecting the resulting mass shift of the protein in SDS-PAGE. Conjugation was done using the heterobispecific crosslinker Sulfo-SMCC, containing a NHS-ester and maleimide group. The maleimide group of Sulfo-SMCC forms disulfide bonds with thiol groups (e.g. SH-modified oligonucleotides or cysteine). The NHS-ester reacts with primary amins present in amino acids to amide bonds. The pH conditions during the conjugation are set to pH 7.2 for stabilizing the NHS-ester. The NHS-conjugation can be hydrolyzed at higher pH-values. Therefore the pH value has to be controlled during the conjugation.

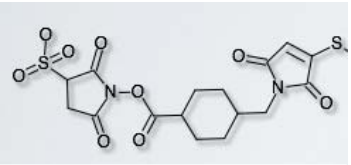


Figure 30: Structure of the heterobispecific crosslinker Sulfo-SMCC

The conjugated primer P1 used in the first assays consists of 35 nucleotides and a thiol group resulting in a molecular mass of 10.5 kDa. P2 with an identical sequence but an additional Cy3 label has an even higher molecular mass of 20 kDa. The mass shift should be easily detectable in SDS-PAGE or other gel systems. Antibodies are delivered with buffers containing Azide, preventing microbial contamination and stabilizers like BSA and gelatin. Both additives can react with Sulfo-SMCC. To circumvent such problems, first tests were performed with recombinant purified proteins (GST-tagged STIP and the His-tagged Filamin A (FilA)). For both proteins high molecular protein bands could be detected after conjugation. Either multiple DNA fragments were conjugated per protein or a cross conjugation of the proteins was the reason for the increased shift. The WB of the proteins showed that the high molecular protein contains in the case of GST-STIP still the GST-tag and in the case of His-FilA still the His-tag. One possible explanation

is that Sulfo-SMCC reacts with the free amins of the protein and the available thiol groups of cysteines. Especially GST-STIP contains free SH-groups that can react with Sulfo-SMCC and results in a crosslink of the proteins.

Both proteins, His-FilA and GST-STIP, show the high molecular aggregates. Therefore the experiments were done with two antibodies; αActin-AB and αGST-AB. αActin-AB was very low concentrated. During the concentration step also all additives were concentrated. For αActin-AB the high molecular aggregates could not be detected after conjugation. Unfortunately also a band indicating the successful conjugation was not detectable. Nevertheless, the antibody was tested for functionality in WB. The unmodified αActin-AB (positive control) was able to recognize 1 pmol actin in cell lysates. The signals for αActin-AB-P1 and αActin-AB incubated with Sulfo-SMCC without primer P1 were less intense compared with the positive control. The assay does not allow discriminating if this is due to a reduced functionality of the antibody or if the used antibodies and the conjugation products have a different concentration.

To show the conjugation a new primer was used. P2 has the same nucleotide sequence as P1, but has an additional Cy3-label between the thiol group and the nucleotides on the 5' end. The conjugation product can now easily be distinguished from possible cross conjugations of proteins / AB's as shown in Figure 17 and Figure 16. After conjugation a clear signal of the conjugated primer P2 at λ_{em} 532 nm in fluorescence scan of a SDS-PAGE could be detected. The yield of conjugation product to not bound AB-chains is lower, compared to results of Lapiene and co-workers (Lapiene, Kukolka et al. 2010). As alternative to Coomassie staining and sensitive method silver staining was used to detect even the low amount of conjugation product as fine bands. Silver staining of SDS-PAGE revealed a lot of impurities in the AB, making it impossible to distinguish the conjugation product from the background. From the gels (Figure 17 and Figure 18) it was estimated that below 5% of the antibody were conjugated with a DNA fragment. For the following functional analysis a purification or concentration step of the conjugated antibody was done. As described by Lapiene (Lapiene, Kukolka et al. 2010) a subsequent gelfitration and anion exchange step, can further increases the purity of the conjugates.

The simplest way to detect the conjugation is a separation by SDS-PAGE. For all proteins tested a shift of the conjugated protein was not detectable. Probably the reducing conditions of the SDS-PAGE could be a reason for breaking the covalent bond of heterobispecific Sulfo-SMCC crosslinker. Either the thiol reactive maleimide functionality or the amino-reactive NHS group could be damaged by β-mercaptoethanol, a compound of the loading dye for the SDS-PAGE. Loading the protein conjugates to SDS-PAGE without denaturation at 95 °C was tested with no difference according to denaturized samples after Coomassie staining of SDS-PAGE. For visualization the molecular mass difference of conjugated proteins native PAGE was introduced. Native PAGE's according to Walker *et al*. (Walker 1994) lead to no clear protein bands in gel. The native PAGE by Walker gave no indication why the bands are not sharp for a single molecule, since the PAGE was performed at 4°C, to avoid denaturation of proteins, due to heat while running the gel. Even the use of a stacking gel did not improve results as recommended by the authors. The improved method of Wittig and colleagues (Wittig, Braun et al. 2006) should overcome this problems. The method was developed to detect proteins from cell lysates, especially membrane bound complexes in the ranging from 10 kDa to 10 MDa (Schagger, Cramer et al. 1994). The 144 kDa AB and the His-FilA could not enter the separation gel. The problem of the limited migration of bigger proteins into the gels could be based on the salt concentrations of the used samples, according to the troubleshooting advises by the authors.

Finally the conjugation of primer P1 and P2 to αGST-AB was successfully established. Before using this conjugation method as standard method optimization is necessary. Several different methods for the conjugation are described in the literature. In a recent publication by Lapiene *et al*. (Lapiene, Kukolka et al. 2010) a swap of the reaction chemistry together with a purification strategy was described. This group conjugates DNA to proteins using the same conjugation reagent (Sulfo-SMCC) but they used an alkylamino modified primer and used the internal thiol groups of protein (e.g. in cysteine). Even under denaturing conditions they achieved to visualize a shift of about 8 kDa in a 14% SDS-PAGE stained with Coomassie. An alternative conjugation method is the biotin-Streptavidin interaction. Biotinylysation of antibodies and Streptavidin modified oligonucleotides are used in an assay published by Schlingemann *et al*. (Schlingemann, Leijon et al. 2010). Biotinylated proteins are

common in cells, therefore an interaction of biotin from samples and the biotin used for conjugation is possible. Other alternative strategies like Click Chemistry are easy and reliable but need organic solvents. The reaction conditions are not suitable for a functional protein. All conjugation protocols rely on the functionalities of amino acids. To increase the labeling efficiency the protocols use an excess of labeling reagent and DNA. This can result in multi labeled proteins. The advantage of multiple labeled antibodies is a higher sensitivity that can be achieved. On the other hand multiple labels on antibodies results in an increased risk that the paratope of the antibody is modified (Figure 31). The possible conjugation of DNA to the paratope of the AB would disrupt the structural target recognition entity of the AB.

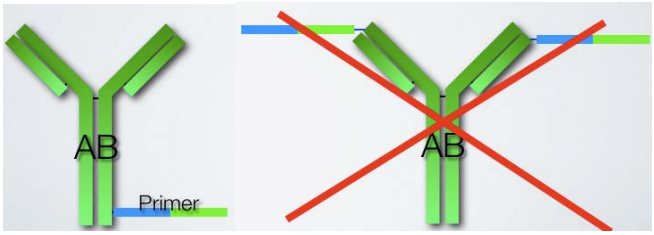


Figure 31: Possible conjugation positions for the oligonucleotide, the conjugation to paratope (right), is invalidated with the positive signals in WB for the used antibodies αGST-AB and αActin-AB.

4.2 Rolling circle amplification (RCA)

The generation of the circular template for the signal amplification is the prerequisite for the RCA. An oligonucleotide that can form a hairpin was chosen. Using the OligoAnalyzer 3.1 by IDT the secondary structures of the used templates are predicted. Although both oligonucleotides have similar ΔG values the resulting structures are slightly different. In the case of O2 the ligation site is outside the double stranded area (Figure 30). This explains the low ligation efficiency.

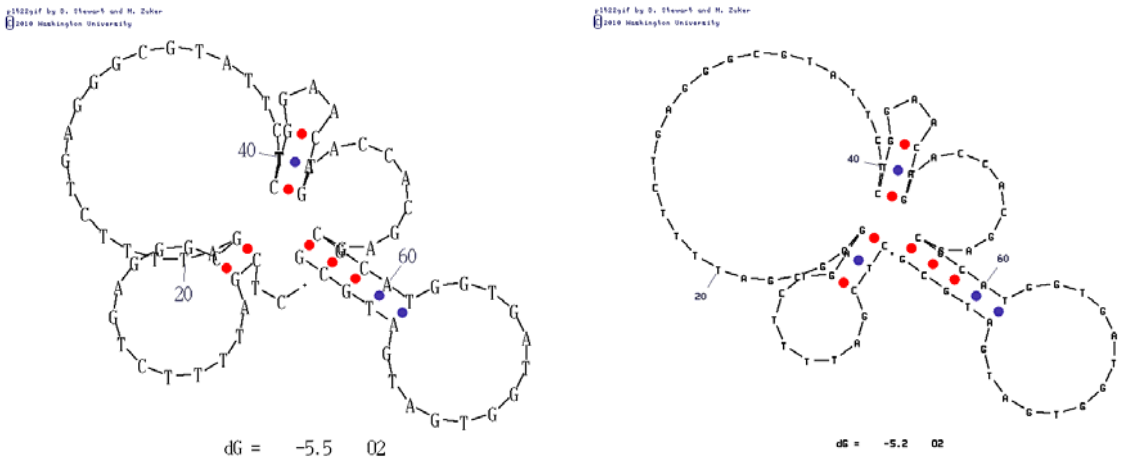


Figure 32: Predictions of secondary structures of the RCA template O2 made by http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/. Even for the second best ΔG value one can see, that a double stranded region is not achieved by forming the internal hairpin structure.

Oligonucleotide O1 can form a stem loop (Figure 33 right). The ligation side is directly in this stem loop. The generation of a circular template for the RCA using the oligonucleotide O1 was successful. The predictions of the secondary structures are in line with the experimentally data.

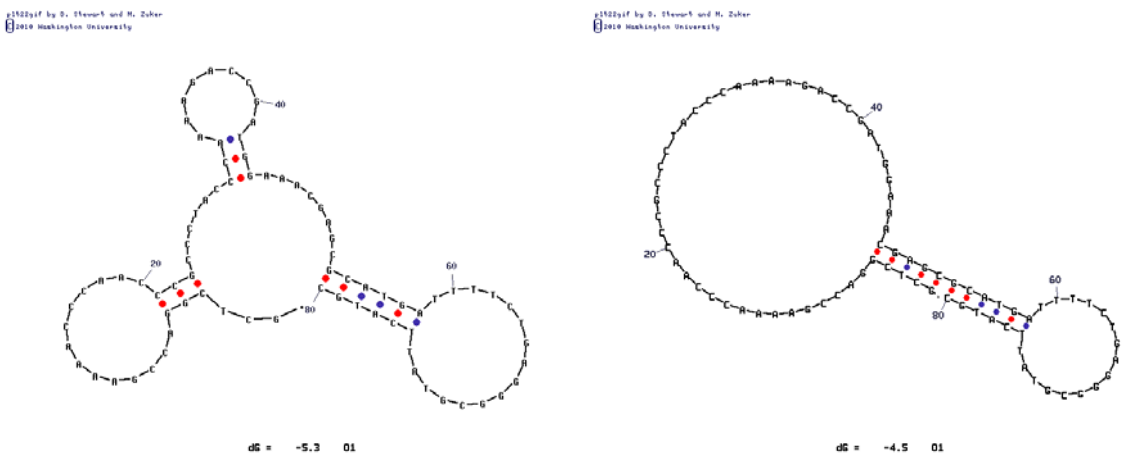


Figure 33: Predictions of secondary structures of the RCA template O1 made by http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/. The left picture shows an internal double stranded region.

The generation of the circular template holds the advantage of skipping one purification step for separation of unbound connector oligonucleotides compared to the template generation used in standard assays, using two oligonucleotides for generation of a double stranded region (Lizardi, Huang et al. 1998).

The generated circular template is used to establish the RCA with the primers P1 and P2. The concatemeric RCA-Product (RCP) is high molecular and can be easily detected in PAGE. In pre-experiments the incorporation of modified nucleotides by Phi29 Polymerase was shown. Those pre-experiments were the basis that allows the adaptation of the method to DNA-antibody conjugates. Best results were obtained with Cy3-dUTP. The RCA product was detectable in PAGE and on the membrane. The results are in line with the RCA amplification in solution.

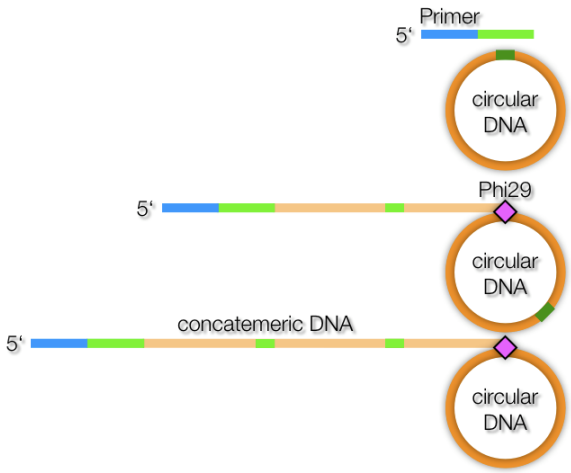


Figure 34: Principle of the RCA and generation of a RCP using a primer and a circular template DNA as starting point for the Phi29-Polymerase

The last part is the establishment of the RCA on the AB bound to membrane.

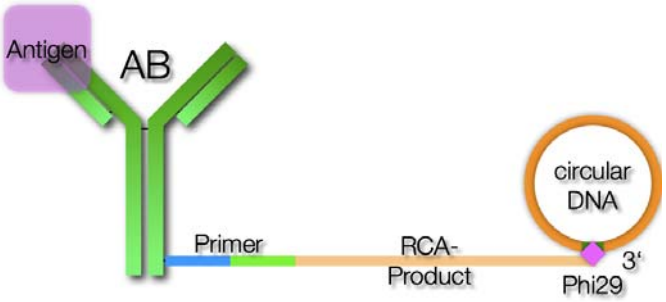


Figure 35: Illustration of the DN-AB conjugate detecting a target and using the DNA-primer and a circular template DNA as starting point for the RCA resulting in DNA amplification as generated signal in immunoassays.

Figure 35 schematically shows the compounded DNA-AB and the use in an immunoassay for detection of low concentrated targets. The pre-hybridized circular oligonucleotide on the DNA-AB conjugate serves as template for the RCA. The resulting rolling circle product (RCP) delivers the signal which is to detect. GST was blotted to nitrocellulose membrane and the blot was developed with the DNA-AB conjugation products, which were pre-hybridized with the circular template DNA for the RCA. The RCA was performed covering the membrane with a layer of RCA solution holding all the needed compounds at 30 °C for 3h at constant humidity. The RCA for direct detection of the RCA incorporates Cy-labelled dUTPs into the RCP (Figure 36).

The results of Soderberg *et al.*, (Soderberg, Gullberg et al. 2006) suggest a good accessibility of the DNA on antibodies. The hybridized DNA-probes used for the RCP detection in their assay, holding a lower K_D as the biotin Streptavidin interaction, but deliver a good signal for validating the RCP on AB, schematically shown in Figure 36 B. The assay has to be optimized, by using pure ingredients. The application of chromatographic separation methods would result in higher concentrated and purified DNA-AB conjugates. The selection of antibody suppliers together with carefully established enrichment strategies should exclude many impurities from AB suppliers and therefore they would definitely result in more conjugation product. The use of Sulfo-SMCC as conjugation reagent is still the matter of choice. It could be possible that free amins and free cysteines are used for conjugation of Sulfo-SMCC to protein (as indicated in the high molecular conjugation products in the preliminary tests) in the first step. The available cysteines in proteins are much less frequent, compared to the available free primary amins.

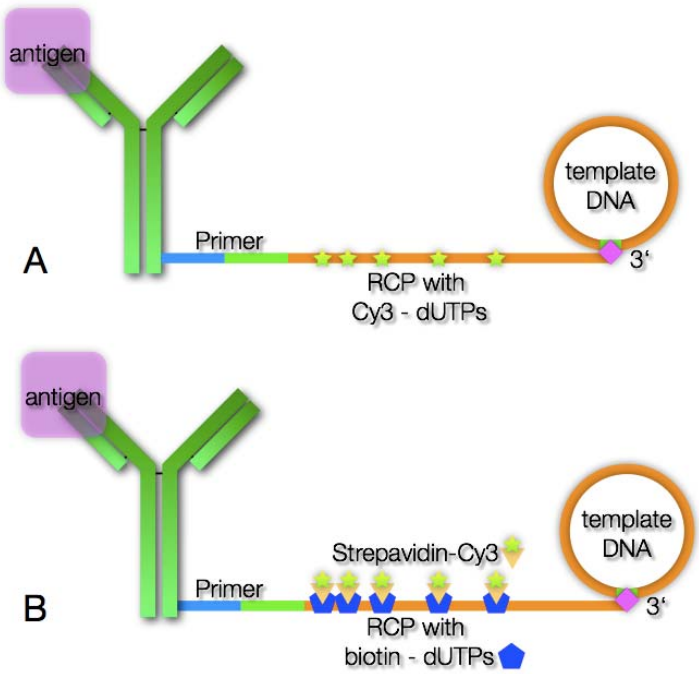


Figure 36: Principle for the detection of RCP, using A: Cy3-labelled dUTPs, or B: biotinylated dUTPs for Cy3-Streptavidin interaction.

The conjugation of DNA to protein using the free primary amins of the proteins for conjuagtion to a thiol group on the primer could successfully be established, using Sulfo-SMCC as a conjugation reagent. The best proof for this conjugation system is the Cy3-labeled Primer for visualizing the shift of the protein bands. The rolling circle amplification for amplification of a circular template DNA could be expanded to DNA-AB conjugation product. Validating the functionality of the AB after conjugation process was positive and subsequent RCA on membranes need to be optimized. The results for incorporation of Cy3-dUTPs in RCP on DNA-AB conjugates give reason to argue for optimization of the method.

5 Summary

Analyzing signaling pathways (SP) in small amount of cells or even in single cells is very challenging. Following information transmitting proteins requires a sensitive assay with outraging detection rates to distinguish modified vs. unmodified proteins. Proteins have to be detected over a wide concentration range. A temporal resolution of protein based answers in SP is needed additionally. The small quantities of proteins used in SP makes it necessary to improve signal amplification in immunoassays by DNA based detection of proteins. The outraging epitope recognition of monoclonal antibodies (AB) is combined with the rolling circle amplification (RCA) for signal amplification. AB's are labeled with short DNA sequences serving as starting points for the RCA. Free primary amines of AB's are used for conjugation to SH modified DNA. Conjugation could successfully be established, using Sulfo-SMCC as a conjugation reagent. Proof of conjugation succeeded with Cy3-labeled primer for visualizing the conjugation product. Readout of immunoassays using the DNA-AB conjugates is switched from a protein based one to DNA based readout. In contrast to established PCR-based signal amplification the RCA works at constant temperatures, not disrupting the non covalent antibody antigene interaction in immunoassays. The concatemeric RCA-Product is a key feature of this assay. The increased mass of DNA on the AB can be detected in multiple ways. A direct way for signal detection is the fluorescent dye incorporation. For indirect detection of the RCP, interaction of labeled probes is the method of choice. Fluorescent labeled Streptavidin interacts with the biotinylated RCP. The RCA for amplification of a circular template DNA could be expanded to DNA-AB conjugation products. The functionality of the AB after conjugation process was validated and subsequent RCA on membranes need to be optimized. The established method can be expanded to biosensing tests for examples in life sciences and more important in healthcare, using the protein microarrays for substance detection only present in small amounts of sample or in high dilutions.

Zusammenfassung

Die Analyse von Signalwegen in wenigen Zellen oder sogar in Einzelzellen ist ein herausforderndes Forschungsfeld. Proteine spielen bei der Signalübertragung eine wichtige Rolle. Eine zeitliche Auflösung der Signalwege ist ein Schlüsselpunkt, um die Informationsvermittlung in lebenden Systemen zu verstehen. Um diese Proteine und damit die Signalwege verfolgen zu können werden sensitive Analysemethoden mit herausragenden Detektionsraten benötigt. Die dafür nötigen Immunoassays werden an zwei Anforderungen gemessen. Zum einen die spezifische Detektion von Proteinen über einen sehr großen Konzentrationsbereich und zum zweiten die zeitliche Auflösung der Signale. Die geringe Konzentration von Proteinen und insbesondere die post translational modifizierten Proteine in Signalwegen, erfordert eine Signalverstärkung. Als Alternative zu klassischen ELISA Assays haben sich dabei in den letzten Jahren Protein Mikroarray basierte Methoden etabliert. Zur Signalverstärkung sind dabei DNA basierte Detektionsmethoden sehr viel versprechend.

Die Kombination mit den herausragenden Eigenschaften von monoklonalen Antikörpern in der Epitoperkennung und der rolling circle amplification (RCA) ist das Ziel dieser Arbeit. Die Konjugation von Antikörpern (AK) mit kurzen DNA Sequenzen ist die Grundlage. Die konjugierten DNA Fragmente dienen als Primer für die am DNA-AK Konjugat stattfindende RCA. Die Konjugation mittels Sulfo-SMCC als Kopplungsreagenz wurde erfolgreich etabliert. Dabei wird ein SH modifizierter Primer kovalent an die freien Amine von Antikörpern gebunden. Die Konjugation konnte mit einer Cy3-Markierung des verwendeten Primers nachgewiesen werden. Das Signal für den Immunoassay ist damit von einem proteinbasiertem zu einem DNA-basierten verändert worden. Die RCA hat gegenüber der PCR-basierten DNA-Amplifikation den Vorteil isotherm zu sein. Eine starke Temperaturerhöhung würde die Interaktion des AK's mit dem zu erkennenden Epitop auflösen und damit wäre das Antigen nicht mehr nachweisbar.

Als Produkt entsteht bei einer RCA ein langes einzelsträngeres DNA Fragment das aus vielen Kopien von dem verwendeten Template besteht. Dieses RCA Produkt (RCP) kann auf unterschiedliche Weisen nachgewiesen werden. Ein direkter Weg ist der Einbau von Fluoreszenzen oder die Färbung der DNA. Indirekt lässt sich das RCP

durch Hybridisierung mit markierten Sonden nachweisen.

Die RCA zur Amplifikation einer zirkulären DNA konnte erfolgreich mit einem DNA-Antikörper Konjugat durchgeführt werden. Die AK-Funktion (Epitoperkennung) konnte noch der Konjugation erfolgreich validiert werden. Der Einbau von Cy3-markierten dUTPs in das RCP am DNA-AK Konjugat konnte gezeigt werden, jedoch benötigt die RCA an dem DNA-Antikörper Konjugat noch weitere Optimierung.

Der etablierte Test kann auf bestehende Analysen z.B in der Landwirtschaft und Tierzucht, aber noch viel mehr im Gesundheitswesen angepasst werden. Die Nutzung von Protein Mikroarrays ermöglicht eine sehr sensitive Erkennung von Substanzen, die nur in Spuren oder geringen Konzentrationen vorkommen.

6 Literature

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Eidesstattliche Erklärung

Hiermit versichere ich an Eides statt, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Hilfsmittel verwendet habe. Alle Abbildungen und Textpassagen, die anderen Werken im Wortlaut entnommen sind, habe ich mit Quellenangaben kenntlich gemacht.

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