Complement immunoassay development for biosafety researches

Summer School Program
Biosafety H2020 Twinning project

14-15.06.2021

Overview

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This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 882830.

Introduction

TECOdevelopment GmbH

Placed in Rheinbach, Germany

Part of the TECOmedical Group

Leading provider of **in-vitro specialty test systems** and instruments in the areas of **medical and veterinary diagnostic, biosafety, vaccines and environmental testing**.

The company expertise also includes the development, validation and test kit production of ELISA based assays under EN ISO 13485.

Introduction

Immunoassays research project

Marie Skłodowska-Curie Actions (MSCA)
European Comission grant program

Individual Fellowship

Individual Fellowships support the mobility of researchers within and beyond Europe. The grant usually covers two years' salary, a mobility allowance, research costs and overheads for the host institution. **Individual researchers** submit proposals for funding in liaison with their planned host organization. **Proposals** are judged on their research quality, the researcher's future career prospects, and the support offered by the host organization.

Introduction

Immunoassays research project

Development of Complement Immunoassays

For complement protein measurements from serum/plasma

- **C3a**
- **TCC** (Terminal complement complex)
- **Bb**

Biosafety experiments with animal models: **pig** (and mouse)

No great variety of porcine (pig) specific complement ELISAs on the market:
Pig complement immunoassays developments

Introduction

Immunoassays research project

Why are they necessary?

Traditional **small molecules** and also **biologics** such as **therapeutic antibodies**, **fusion proteins** and novel formulations using **liposomes** or **nanotechnology** all can trigger (sometimes severe) **adverse events connected to complement activation** (non-IgE connected, pseudoallergic reactions).

In vivo biosafety studies during the non-clinical development phase is necessary:
Pig immunotoxicology experiments

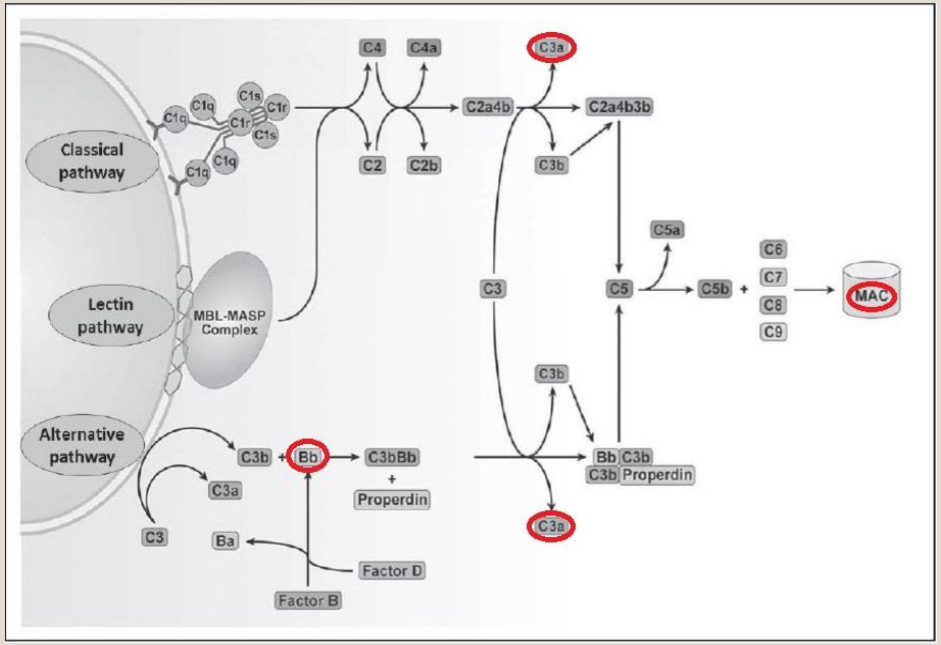
Validated complement assay systems for nonclinical studies in laboratory animals are very limited

Introduction

Immunoassays research project

Complement (C) activation biomarkers

Activation of C system during in vivo biosafety experiment



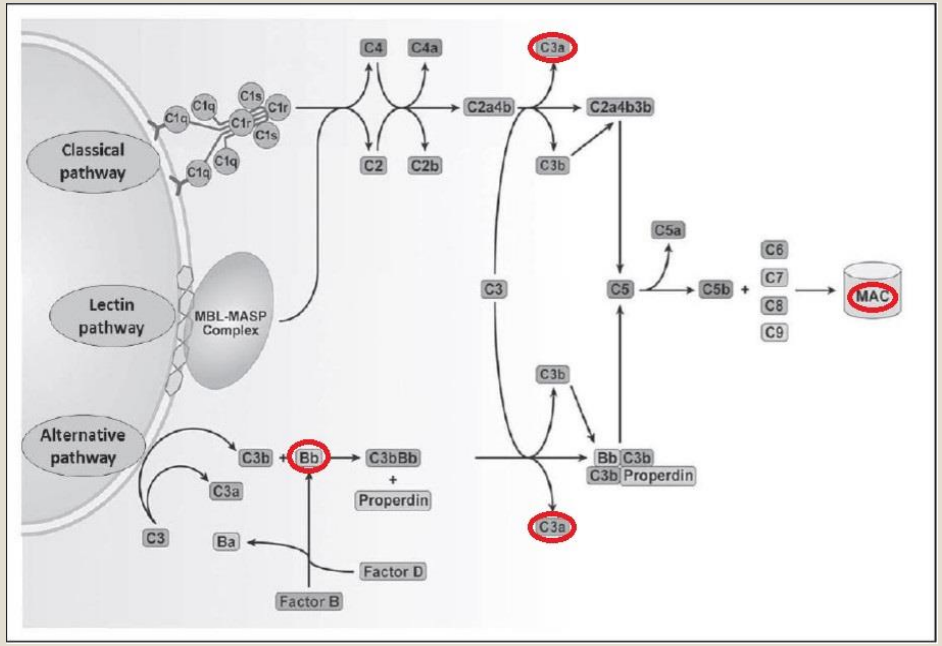
Plasma samples → C component measurements

Introduction

Immunoassays research project

C3a

- Cleavage of complement component 3 to C3a and C3b
- C3a is an **anaphylatoxin** that binds to C3a receptor (G-protein coupled rec)
- **Central role** in all C activation pathways



Introduction

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C3a roles:

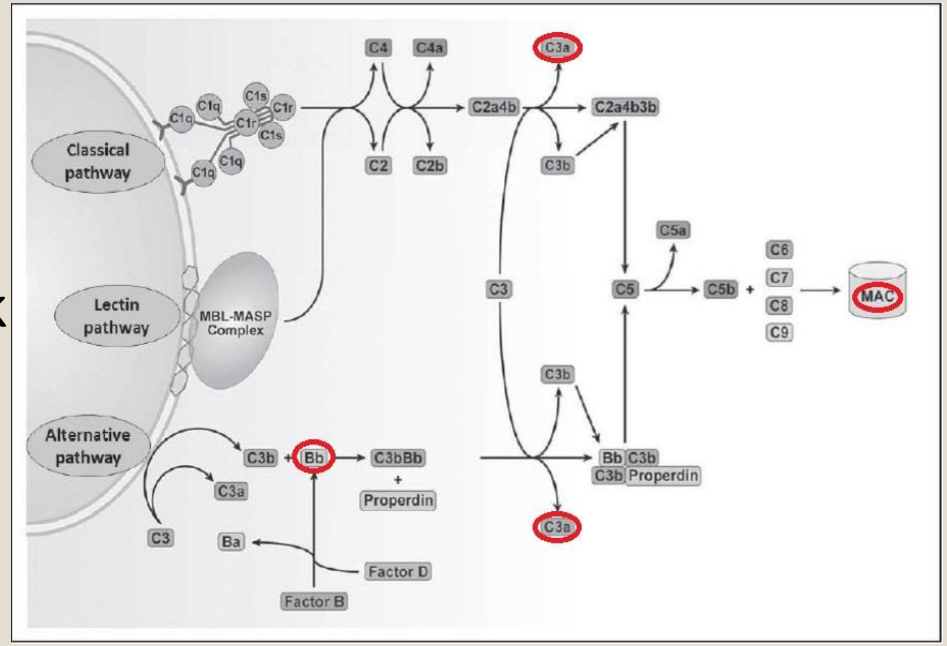
- T cell activation
- Macrophage activation
- Mast cell degranulation (allergy, anaphylaxis)
- Vasodilation
- Chemotaxis (attraction of leukocytes to site)

Introduction

Immunoassays research project

TCC (MAC)

- Terminal complement complex or Membrane attack complex
- **“End product”** of the C activation pathways
- Built up of **C5b, C6, C7, C8** and **C9**
- Forming **membranes** on surfaces or in soluble form (sC5-b9)



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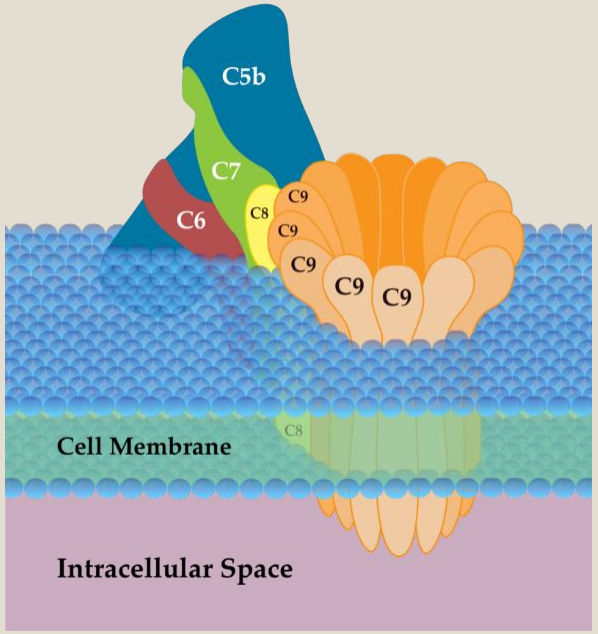
TCC (MAC) roles:

- **Pore formation** to disrupt cell membrane leading to lysis and death of targeted cell
- **Soluble form** can also be produced and can be measured from plasma to indicate the C activation happened



MAC by SLiva2016

Introduction

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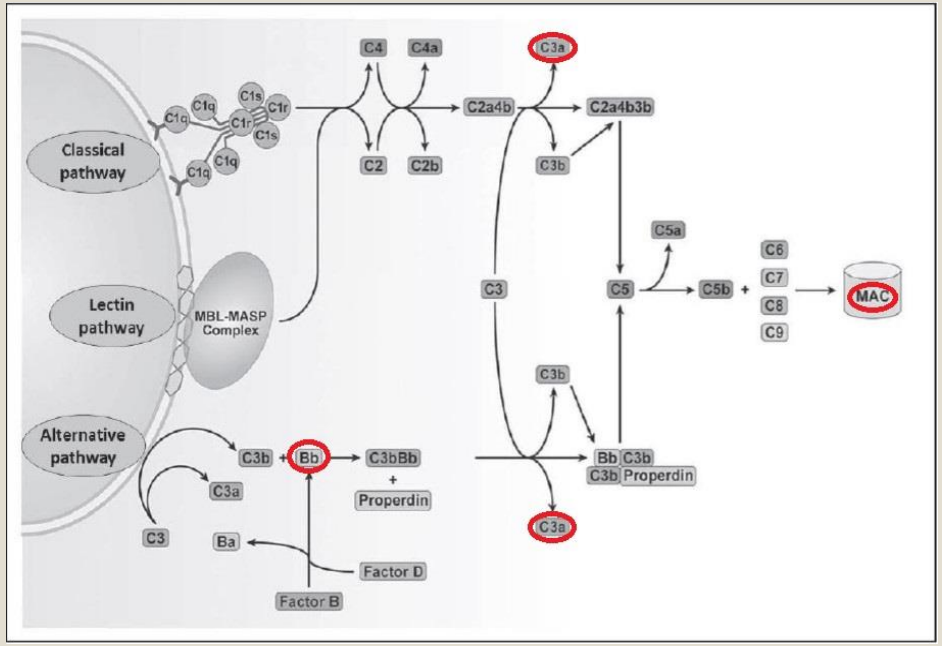
Bb

- Part of the **alternative pathway** C activation
- Cleaved to Ba and Bb by factor D
- Presence in plasma **proves** C activation by alternative pathway during animal biosafety experiment



Introduction

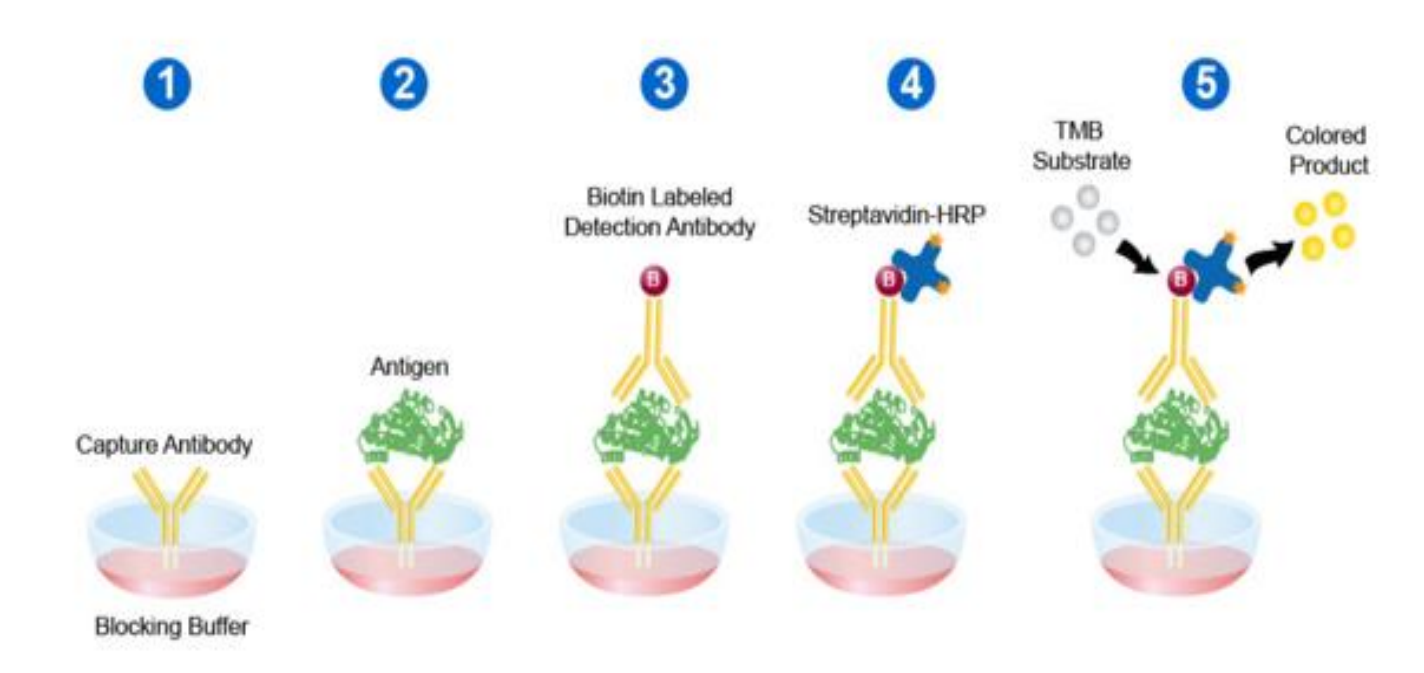
Immunoassays research project

Bb roles:

- Combined with C3b forms **C3a convertase** (C3bBb)
- Propagating C activation
- Proliferation and differentiation of pre-activated B cells

Immunoassays Project

Pig C component specific ELISA development



https://www.leinco.com/sandwich-elisa-protocol/ (accessed Mar. 01, 2021)

Immunoassays Project

Factors to be considered in ELISA development:

- Specific antibody development
- Coating and biotinylated AB concentrations
- Coating, blocking and plate stabilization factors
- Buffers: Type, pH, salts and proteins concentration, addition of serum, etc.
- Incubation: time periods and temperatures
- Washing steps
- SA-HRP (Streptavidin-horseradish peroxidase conjugate): concentration and buffer

Immunoassays Project

Specific ABs for pig complement ELISAs:

- Choosing possible/favourable **peptide sequences** for epitopes: epitope mapping
- Generating peptide specific polyclonal ABs **using rabbit models**
- During immunization (repeated for few months) **bleedings** are used for AB collection via affinity/size exclusion **chromatography**

Immunoassays Project

Pig C3a ELISA development:

Specific AB found on market: Kerafast Inc. Boston USA

- **Anti-Complement C3a/C3a (desArg)/C3 [K5/9] Antibody**
- **Anti-Complement C3a/C3a (desArg) [Z22/8] Antibody**

Mouse IgG2b/k monoclonal antibody was generated against purified porcine complement C3a and recognizes porcine complement C3a/C3a (desArg).

Immunoassays Project

Pig C3a ELISA development:

Biotinylation of ABs

- Covalently binding of the relatively small (MW 244,31g/mol) biotin molecules to the detecting antibodies
- Chemical labeling method is used
- Biotinylated ABs are stored with 50% Glycerin at -20°C

Immunoassays Project

Pig C3a ELISA development:

AB concentrations

Serum dilutions			1	2	
PIG 1	1:100	A	a	c	Biot AB1 a: 25ng/ml b:12,5ng/ml c:6,25ng/ml d:3,13ng/ml
	1:100	B			
	0	C			
	0	D			
	1:100	E	b	d	
	1:100	F			
	0	G			
	0	H			
			Plate coating: AB2 50ng/ml		

Serum dilutions			1	2	
PIG 1	1:100	A	0,713	0,192	
	1:100	B	0,706	0,175	Biot AB1
	0	C	0,245	0,204	a: 25ng/ml
	0	D	0,196	0,160	b:12,5ng/ml
	1:100	E	0,203	0,143	c:6,25ng/ml
	1:100	F	0,211	0,149	d:3,13ng/ml
	0	G	0,183	0,160	
	0	H	0,182	0,162	
			Plate coating: AB2 50ng/ml		

Two stripes were coated with 50ng/ml AB2, 100µl/well. After overnight incubation on 4-8°C, plate coating was blocked with washing buffer (BUF01) 200µl for 1h, then 5x wash of all wells was prepared.

- 75µl matrix (BUF02)
- 25µl/well diluted pig 1 serum diluted with dilution buffer (BUF02) 1:100
- 1h incubation on RT on shaker
- 3x wash with wash buffer
- **100µl/well biotinylated AB1 (25ng/ml, 12,5ng/ml, 6,25ng/ml and 3,13ng/ml)**
- Incubate 2h RT on shaker
- Wash 3x
- Add 100µl/well SA-HRP (conc. 100ng/ml) diluted with TP2 (1x)
- Incubate 30min on RT on shaker
- Wash 5x
- Add 100µl TMB
- Incubation 30min on RT on shaker

Stop solution 100µl/well

Conclusion: Decreasing the biotinylated AB decreases too much the ODs of the samples. **25ng/ml** seems to be the best setup.

Further actions: Try different coating and blocking buffers.

Immunoassays Project

Pig C3a ELISA development:

Coating buffer and blocking

			Biot AB1 25ng/ml		
Serum dilutions			1	2	Coating blocking
PIG 1	1:200	A	3,607	0,899	a: Wash buffer
	0	B	0,128	0,084	
	1:200	C	2,993	2,448	b: 2%BSA buffer
	0	D	1,690	2,489	
	1:200	E	3,723	2,934	c: 2%Casein buffer
	0	F	0,189	0,150	
	1:200	G	2,355	1,210	d: 1%gelatine + 20%glucose
	0	H	0,114	0,100	
			Plate coating: AB2 100ng/ml		
			with 1/10PBS	with BUF03	

To compare **coating buffers**, 100ng/ml AB2 was diluted in PBS 1/10 and coating buffer (BUF03) using two stripes. After overnight incubation on 4-8°C, **plate coating was blocked** with washing buffer (BUF01), 2%BSA buffer (BUF04), 2%Casein buffer (BUF05) and 1%gelatine-20%glucose buffer (BUF06), 200µl/well for 1h, then 5x wash of all wells.

- 75µl matrix (BUF02)
- Standard: Pig1 (diluted with buffer BUF02) 25µl/well (or 25µl BUF02 for blank)
- Incubate 1h on RT on shaker
- 3x wash (BUF01)
- Add 25ng/ml biotinylated AB1 (dilution buffer BUF02) 100µl/well
- Incubate 2h on RT on shaker
- 3x wash (BUF01)
- Add 200ng/ml SA-HRP (in TP2 (1x)) 100µl/well
- Incubate 30 min on RT on shaker
- 5x wash (BUF01)
- Add TMB 100µl/well
- Incubate 30min on RT on shaker
- Stop buffer 100µl/well

Conclusion: For blocking the **2%casein buffer** together with **PBS1/10 coating** produced the best color.

Further actions: Use the combination of PBS1/10 buffer for coating and 2%casein buffer as blocking buffer. Try different streptavidin-HRP (SA-HRP) buffers.

Immunoassays Project

Pig C3a ELISA development:

SA-HRP buffer test

			SA-HRP 100ng/ml	SA-HRP 200ng/ml	
Serum dilutions			1	2	
PIG 1	1:200	A	1,804	2,067	a: SA-HRP + TP2 (1x) Ref
	0	B	0,060	0,066	
	1:200	C	0,860	1,120	b: SA-HRP + SA1 buffer
	0	D	0,038	0,053	
	1:200	E	0,950	1,383	c: SA-HRP + SA3 buffer
	0	F	0,049	0,055	
	1:200	G	1,389	1,807	d: SA-HRP + Noble buffer
	0	H	0,026	0,049	
			Plate cover: AB2 50ng/ml		
			Biot AB1 25ng/ml		

After plates were coated with 50ng/ml coating AB and incubated overnight on 4-8°C, coating was blocked with 200µl 2,5% casein buffer for 1H, then 5x wash all wells with wash buffer.

- 75µl matrix (BUF02)
- Standard: Pig1 (diluted with BUF02) 25µl/well (or 25µl dilution buffer for blank)
- Incubate 1h on RT on shaker
- 3x wash (BUF01)
- Add 25ng/ml biotinylated AB1 (diluted with BUF02) 100µl/well
- Incubate 2h on RT on shaker
- 3x wash (BUF01)
- Add **100 or 200ng/ml SA-HRP diluted in different buffers**: in a) TP2 (1x), b) SA1 buffer, c) SA3 buffer and d) Noble buffer. 100µl/well
- Incubate 30 min on RT on shaker
- 5x wash (BUF01)
- Add TMB 100µl/well
- Incubate 30min on RT on shaker
- Stop buffer 100µl/well

Conclusion: **200ng/ml of SA-HRP diluted with Noble buffer** seems the better option, produced better color with lower background.

Further actions: Use 200ng/ml SA-HRP diluted with Noble buffer.

Immunoassays Project

Pig C3a ELISA development:

Incubation time and temp.

			Incubation times: **first step** (STD)-**second step**(Biot-AB)-**third step**(HRP) Incubation tempereatures: **C**: cold **R**:room temperature				
			C-R-R	**C-R-C**	**C-C-R**	**R-C-C**	**R-C-R**
Serum dilutions			2	3	4	5	6
2H : 2H	1:200	A	1,551	1,274	3,415	3,663	3,576
	1:200	B	1,565	1,260	3,561	3,603	3,624
	0	C	0,158	0,107	0,066	0,112	0,093
	0	D	0,151	0,120	0,061	0,103	0,093
1H : 1H	1:200	E	1,614	1,310	2,157	2,971	2,727
	1:200	F	1,649	1,292	2,175	2,919	2,802
	0	G	0,128	0,085	0,052	0,104	0,088
	0	H	0,139	0,079	0,061	0,106	0,062
			Plate coating: AB2 25ng/ml				
			Biot AB1 25ng/ml				

			C-C-C
Serum dilutions			1
2H : 2H Stationary	1:200	A	3,309
	1:200	B	3,364
	0	C	0,077
	0	D	0,085
2H : 2H On shaker	1:200	E	3,802
	1:200	F	3,759
	0	G	0,119
	0	H	0,131

After an over weekend coating on 4-8°C, coating was blocked with 2,5% casein buffer, 200µl added on top of the coating buffer and incubated for 1h, then 5x wash all wells with wash buffer.

New dilution buffer is used without containing BSA: PBS-T buffer (BUF02 without BSA)

- 75µl matrix (BUF02)
- Standard: Pig1 (in 50%glycerin) diluted to 1:200, 25µl/well (or 25µl BUF02 for blank)
- **Incubate 2h or 1h on 4°C or on RT with shaker (or without shaker)** (see table)
- 3x wash
- Add 25ng/ml biotinylated AB1 diluted with dilution buffer BUF02 100µl/well
- **Incubate 2h or 1h on RT or on 4°C with shaker (or without)** (see table)
- 3x wash
- Add 200ng/ml SA-HRP diluted with Noble buffer. 100µl/well
- **Incubate 30 min on 4°C or on RT on shaker**
- 5x wash (BUF01)
- Add TMB 100µl/well
- Incubate 30min on RT on shaker
- Stop buffer 100µl/well

Conclusion: No major difference between plates incubated on shaker or stationary. **2 Hours-2 hours** (for standard step and biotinylated AB step, HRP step is kept on half an hour always) incubation time protocol produced better colors. **Biotinylated AB step** in the procedure should be definitely incubated **on 4°C**.

Further actions: Standard and Biotinylated AB steps in the procedure should be incubated for 2H-2H, with the standard incubation on RT on plate shaker, the biotinylated AB incubation on 4°C stationary. The HRP incubation step is kept for 30min on RT on plate shaker.

Immunoassays Project

Pig C3a ELISA development:

Matrix buffer pH tests

		Matrix with TBS-T				Matrix with PBS-T
STD		pH 8	pH 8,5	pH 9	pH 9,5	pH 7,35
1:200	A	1,278	1,416	1,535	1,588	1,255
1:400	B	0,744	0,880	0,840	0,825	0,793
1:800	C	0,459	0,480	0,524	0,453	0,456
1:1600	D	0,300	0,275	0,304	0,252	0,264
1:3200	E	0,175	0,168	0,164	0,186	0,138
1:6400	F	0,117	0,107	0,107	0,118	0,106
1:12.800	G	0,085	0,085	0,079	0,110	0,086
0	H	0,054	0,053	0,057	0,101	0,093
		Plate coating: AB2 15ng/ml				
		Biot AB1 15ng/ml				

Five stripes of 15ng/ml overnight coated plate were blocked Casein 2,5% buffer: 200µl, 1 hour, RT. Afterwards blocked with wash buffer, washed 5 times before use.

- **75µl matrix with different pH Tris-buffered saline (TBS-T)** (BUF07) prepared, **with PBS-T used as reference**, to check if more basic pH gives better color with lower background.
- Standard: Prepared from reference STD diluted in PBS-T for standard curve from 1:200 to 1:12.800. 25µl/well. Incubate 2h on 4°C.
- 3x wash
- Add 15ng/ml biotinylated AB1, 100µl/well
- Incubate 2h on 4°C
- 3x wash
- Add 200ng/ml SA-HRP diluted with Noble buffer. 100µl/well
- Incubate 30 min on RT, on 4°C
- 5x wash
- Add TMB 100µl/well
- Incubate 30min on RT on shaker
- Stop buffer 100µl/well

Conclusion: To check a more basic pH buffer for matrix, the previous BUF02 was changed to TBS-T (Tris buffered saline can maintain more basic pH). **TBS-T with pH 8,5 as matrix buffer** produced good color, good variation between the standard points with low blank value.

Further actions: Use TBS-T (BUF07) with 8,5pH as matrix and STD dilution buffer further on.

Immunoassays Project

Pig C3a ELISA development:

Matrix buffer additives and incubation temp tests

	Matrix with TBS 1x pH 8,5							
STD	Reference		Casein 0,5%		Casein 0,5% + 50mM EDTA		50mM EDTA	
1:200	3,094	2,978	2,654	2,593	2,012	1,954	2,202	2,061
1:400	2,122	2,120	1,771	1,720	1,263	1,262	1,401	1,389
1:800	1,322	1,310	1,094	1,092	0,786	0,770	0,884	0,824
1:1600	0,783	0,784	0,678	0,674	0,464	0,465	0,484	0,579
1:3200	0,437	0,439	0,378	0,378	0,265	0,275	0,280	0,265
1:6400	0,246	0,249	0,226	0,223	0,156	0,155	0,161	0,146
1:12.800	0,143	0,145	0,141	0,139	0,104	0,103	0,105	0,095
0	0,041	0,043	0,055	0,047	0,048	0,050	0,065	0,044
	Plate coating: AB2 30ng/ml							
	Biot AB1: 15ng/ml							

		All incubations 4°C		Biot-AB incubation on 4°C	
		on shaker	stationary	stationary	on shaker
STD		1	2	3	4
1:200	A	3,127	2,804	3,108	3,499
1:200	B	3,139	2,810	3,201	3,664
1:200	C	3,008	2,805	3,152	3,458
1:200	D	3,105	2,740	3,149	3,518
0	E	0,053	0,045	0,036	0,056
0	F	0,052	0,034	0,034	0,066
0	G	0,054	0,042	0,031	0,049
0	H	0,053	0,047	0,028	0,044
		Plate coating: AB2 30ng/ml			
		Biot AB1 15ng/ml			

Whole plate coated with 30ng/ml AB, overnight incubated at 4°C, was blocked with BUF05 for 1 hour, RT, 200µl/well. Afterwards plate was washed 5 times with wash buffer.

- 75µl matrix with **8,5 pH TBS-T** (BUF07) added to stripe 1 and 2 (Reference); **TBS-T with 0,5% Casein** in stripes 3, 4; **TBS-T with Casein 0,5% + EDTA 50mM** to 5 and 6; **TBS-T with 50mM EDTA** in stripes 7 and 8. For incubation test TBS-T (Reference) as matrix used.
- Standard: Reference STD was diluted to 1:200 then in serial dilution up to 1:12.800 (and 0 for blank) in TBS-T 1x and 25µl added to the wells. Incubate 2h on 4°C, stationary. For incubation test, see table.
- 3x wash
- Add 15ng/ml biotinylated AB1 in Noble, 100µl/well
- Incubate 2h on 4°C (for incubation test see table)
- 3x wash
- Add 200ng/ml SA-HRP diluted with Noble buffer. 100µl/well
- Incubate 30 min on 4°C (for incubation test see table)
- 5x wash
- Add TMB 100µl/well
- Incubate 30min on RT on shaker
- Stop buffer 100µl/well

Conclusion: Adding Casein and EDTA seems necessary for matrix buffer, they produced great variety between the standard points with relatively low blank values. However, the concentrations seem a bit high, **20mM EDTA + 0,1% Casein in TBS-T** can be a good option. According to the incubation test, not all the steps to be incubated on 4°C is necessary, but the **biotinylated AB step definitely should be incubated on 4°C**, not necessarily on shaker.

Immunoassays Project

Pig C3a ELISA development:

Plate coating stabilization test

		1% Gelatine + 10% Sacc.		1% Gelatine + 20% Sacc.		10% Saccharose		20% Saccharose		10%Maltose		20% Maltose		
STD		4°C	RT	4°C	RT	4°C	RT	4°C	RT	4°C	RT	4°C	RT	
1:200	A	3,311	3,164	3,235	3,121	3,155	2,967	3,128	2,951	2,833	2,729	2,928	2,769	Prewash 2min
	B	3,321	3,215	3,343	3,203	3,118	3,023	3,137	3,034	2,988	2,709	2,991	2,872	
0	C	0,079	0,069	0,075	0,077	0,065	0,071	0,056	0,072	0,064	0,074	0,089	0,108	
	D	0,072	0,075	0,076	0,086	0,072	0,066	0,063	0,075	0,073	0,130	0,097	0,125	
1:200	E	3,232	3,197	3,204	3,326	3,187	2,890	3,205	3,188	2,916	2,825	2,984	2,862	without Prewash
	F	3,225	3,213	3,260	3,293	3,200	2,923	3,153	3,036	2,904	2,787	2,917	2,862	
0	G	0,083	0,078	0,076	0,081	0,076	0,063	0,069	0,073	0,068	0,078	0,103	0,107	
	H	0,080	0,074	0,070	0,072	0,063	0,055	0,052	0,064	0,055	0,064	0,080	0,070	
		Plate coating: AB2 30ng/ml												
		Biot AB 1: 15ng/ml												

		10% Glucose		20% Glucose		without stab. Buffer		
STD		4°C	RT	4°C	RT	4°C	RT	
1:200	A	3,206	2,887	3,146	2,929	2,624	2,293	Prewash 2min
	B	3,248	2,794	3,101	2,952	2,830	2,459	
0	C	0,067	0,058	0,061	0,061	0,072	0,052	
	D	0,069	0,058	0,068	0,063	0,060	0,056	
1:200	E	3,135	2,843	3,097	2,965	3,058	2,347	without Prewash
	F	3,150	2,808	3,071	2,948	3,017	2,238	
0	G	0,075	0,068	0,075	0,072	0,062	0,061	
	H	0,069	0,064	0,070	0,064	0,054	0,055	
		Plate coating: AB2 30ng/ml						
		Biot AB 1: 15ng/ml						

Plates coated with 30ng/ml AB2, incubated overnight. Coating was blocked with BUF05 buffer, 1 hour on RT. Then plates were washed with wash buffer five times.

If ELISA plates aren't used right away, they are usually **stabilized with stabilization buffer** and dried for later use. Naturally, a stabilized plate should bring similar values as a freshly coated one.

Different stabilization buffers were added (see setup table), 200µl/well, incubated for 1H on RT. Then the buffers were aspirated, leaving a thin layer on the bottom of the wells. Plates were positioned over plates filled with 30g silica beads and stored to dry on 4°C and RT (dark) for four days.

- Half stripe each prewashed for 2min with wash buffer (280µl per well)
- 75µl matrix (BUF07) with 0,1% Casein (Puf-102) + 20mM EDTA
- Standard: STD was diluted to 1:200 (and 0 for blank) in BUF07 1x and 25µl added to the wells.
- Incubate 2h, RT, on shaker.
- 3x wash
- Add 15ng/ml biotinylated AB1 in Noble, 100µl/well
- Incubate 2h on 4°C, stationary
- 3x wash
- Add 200ng/ml SA-HRP diluted with Noble buffer. 100µl/well
- Incubate 30 min, RT, shaker
- 5x wash
- Add TMB 100µl/well
- Incubate 25min on RT on shaker
- Stop buffer 100µl/well

Conclusion: The **two minutes prewash** seems to be a necessary step. No huge differences between stabilizer buffers, however **1% gelatin with 20% saccharose** seems to be the best option (nice color with low background), **with 4°C storage while plate drying**.

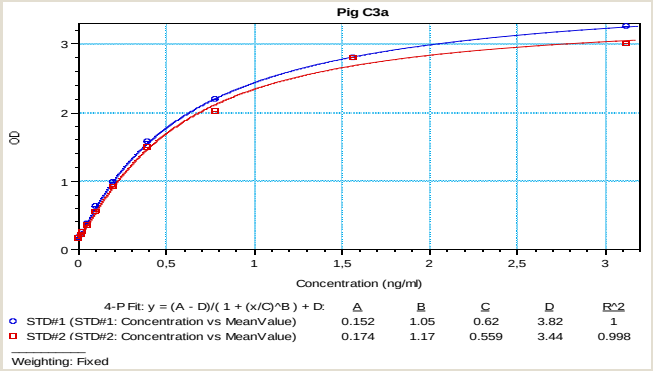
Immunoassays Project

Pig C3a ELISA development:

Standard points test

	Dilutions of "pig pool standard"		
A	1:12,5	1:3200	1:200
B	1:25	1:6400	1:400
C	1:50	1:12.800	1:800
D	1:100	0	1:1600
E	1:200	1:12,5	1:3200
F	1:400	1:25	1:6400
G	1:800	1:50	1:12.800
H	1:1600	1:100	0
	Plate coating: AB2 30ng/ml		
	Biot AB1: 15ng/ml		

	Dilutions of "pig pool standard"		
A	2,975	0,375	2,022
B	3,368	0,250	1,500
C	3,261	0,215	0,914
D	2,798	0,171	0,554
E	2,194	2,667	0,354
F	1,581	2,983	0,256
G	0,977	3,005	0,215
H	0,635	2,796	0,163
	Plate coating: AB2 30ng/ml		
	Biot AB1: 15ng/ml		



Three stripes of 30ng/ml AB2 coated plate was washed with wash buffer for 2 minutes before use.

- 75µl matrix (BUF07) with Casein 0,1% (Puf-102)+ EDTA 20mM was added to the wells
- **Standard: Pig pool standard was diluted** to 1x12,5 then diluted 1:1 eleven times in BUF07 and 25µl added to the wells (Standard 1 and standard 2) Incubate 2h; RT, on shaker.
- 3x wash
- Add 15ng/ml biotinylated AB1 diluted with Noble buffer, 100µl/well
- Incubate 2h on 4°C
- 3x wash
- Add 200ng/ml SA-HRP diluted with Noble buffer. 100µl/well
- Incubate 30 min; RT; on shaker
- 5x wash
- Add TMB 100µl/well
- Incubate 30min on RT on shaker
- Stop buffer 100µl/well

Conclusion: Standard points were measured in duplicates, standard curves were significantly close and showing **optimal values**. Standard curve can be divided into **measurable units**.

Further actions: Units are set for the standard curve planned to be obtainable from the standard stock solution in the ELISA kit: from the pig serum pool a **dilution of 1:100**, which shall be the **highest standard point**, is set to be **500 units/ml**, with 1 unit equaling 1ng/ml. Serum and plasma samples to be measured by the kit can be generally diluted 1:200 to be in range.

Immunoassays Project

Pig C3a ELISA development:

Standard lyophilization test

STD				
1:100	A	PBS-T + 20% sacharose	Lyo. buffer + 1% casein	Blank (w.o. standard)
1:100	B			
1:200	C			
1:200	D			
1:100	E	Lyo. Buffer + 2% BSA	BUF08	
1:100	F			
1:200	G			
1:200	H			
		Plate coating: AB2 30ng/ml		
		Biot AB 1: 15ng/ml		

STD				
1:100	A	2,408	2,456	0,249
1:100	B	2,358	2,403	0,238
1:200	C	1,894	1,916	0,235
1:200	D	1,872	1,788	0,203
1:100	E	2,313	2,314	
1:100	F	2,306	2,261	
1:200	G	1,898	1,892	
1:200	H	1,942	1,868	
		Plate coating: AB2 30ng/ml		
		Biot AB 1: 15ng/ml		

Four **lyophilization buffers** were prepared:

- Lyophilizationbuffer with BSA 2%
- Lyophilizationbuffer with Casein 1% (instead of BSA)
- PBS-T buffer with extra 20% saccharose
- Lyophilization buffer (BUF08)

Pig pool serum was diluted in these buffers, 25µl in each (1:10 diluted). Lyophilized samples were diluted to 250µl (1:100) and diluted further to 1:200 with dilution buffer before use.

Stripes were prewashed 2min with wash buffer (280µl per well)

- 75µl matrix (BUF07) + 0,1% Casein + 20mM EDTA
- **Standards (lyophilized pig pool standards with different buffers)** diluted with BUF07 25µl added to the wells.
- Incubate 2h, RT, on shaker.
- 3x wash
- Add 15ng/ml biotinylated AB1 in Noble, 100µl/well
- Incubate 2h on 4°C, stationary
- 3x wash
- Add 200ng/ml SA-HRP diluted with Noble buffer. 100µl/well
- Incubate 30 min, RT, shaker
- 5x wash
- Add TMB 100µl/well
- Incubate 30min on RT on shaker
- Stop buffer 100µl/well

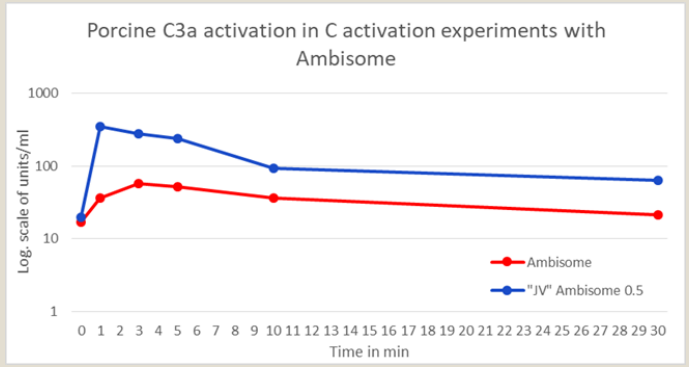
Conclusion: There are no major differences between the effectiveness of the lyophilization buffers, but **the lyophilization buffer with 2% BSA** seems to be a good option.

Immunoassays Project

Pig C3a ELISA development:

Pig experiment plasma measurements

Example for "**positive response**" activation of porcine C3a during C activation experiment:



Example for "**neutral response**" no activation of porcine C3a during C activation experiment:

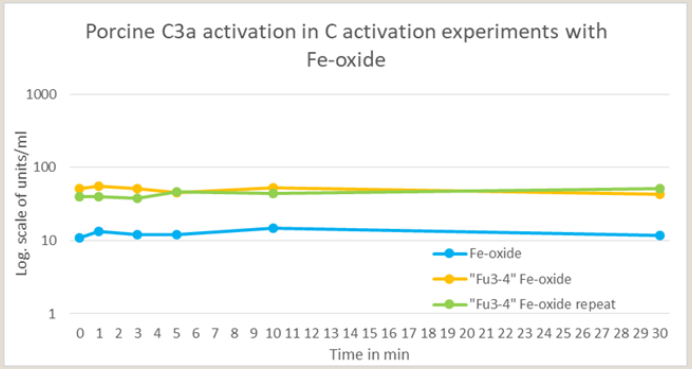


Plate coated with 30ng/ml AB; Stripes were prewashed before use for 2min with wash buffer 280µl per well

- 75µl matrix (BUF07+ 0,1% Casein + 20mM EDTA)
- Standards/Samples were diluted in dilution buffer (BUF07)
- For standard curve lyophilized standard (STD) (LOT 010321) diluted with 500µl dilution buffer
- Pig plasma samples were diluted 1:10
- Incubate 2H, RT, on shaker.
- 3x wash
- Add 15ng/ml biot-AB diluted with Noble buffer. 100µl/well
- Incubate 2H on 4°C, stationary
- 3x wash
- Add 200ng/ml SA-HRP diluted with Noble buffer. 100µl/well
- Incubate 30 min, RT, shaker
- 5x wash
- Add TMB 100µl/well

After 30min incubation on RT, stop enzyme with 100µl/well stop solution

Conclusion: Dilution of the samples 1:10 was sufficient. The clearest and most dramatic **C3a activation** upon administration occurred during the **Ambisome** experiments. In the three **Ferrous oxide** experiments C3a levels were quite steady all through, **no significant C3a activation** was observable.

Immunoassays Project

Pig TCC and Bb ELISA development:

TCC:

- Immunization of rabbits started in October, eight rabbits
- **First Bleeding** sera received in January, Protein A (affinity) chromatography was used to purify Abs. ELISA development concluded that **ABs not specific enough yet**
- From **second bleeding** antigen-specific affinity chromatography of selected ABs arrived, development still in progress
- **Third bleeding** arrived in the beginning of June, sera will be Protein A and also specific affinity purified

BB:

- Immunization of rabbits started in March, three rabbits
- **First bleeding** happened at the end of May
- Development started

Conclusions

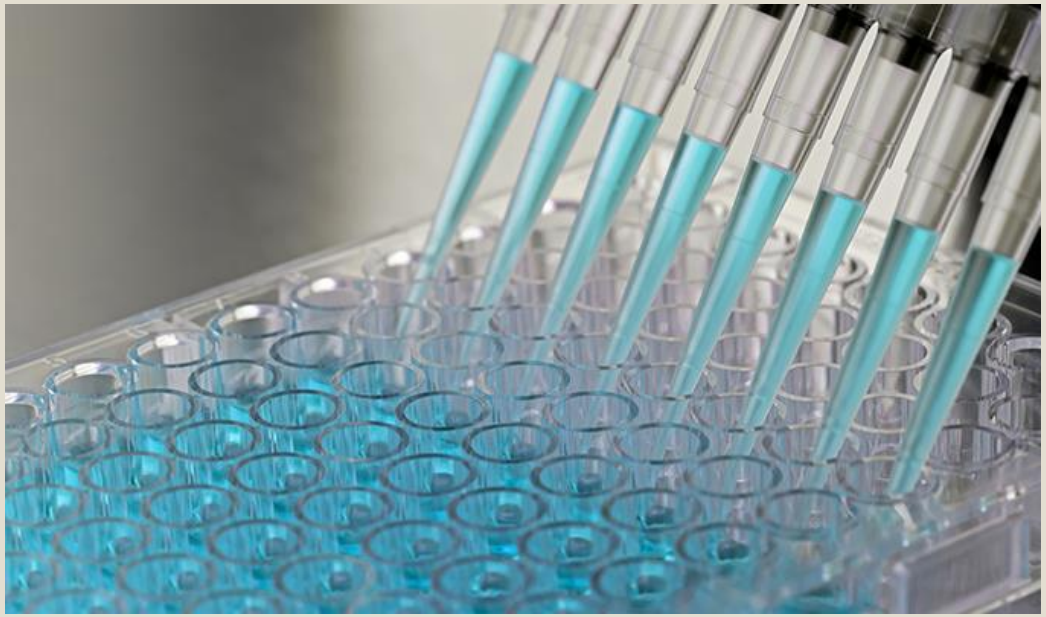
Pig C3a ELISA development:

- **Porcine C3a specific ELISA development was a success using ABs available on the market**
- **Validation phase currently going: stability tests, quality tests and documentation**

Pig TCC and Bb ELISA development:

- **Immunization of the rabbits ongoing**
- **Development to be continued**

Thank you for your attention !!!



Appendix

Buffers compilation (C3a development):

BUF01: Washing buffer with PBS and Tween20 2,5%

BUF02: Sample dilution buffer with PBS, BSA 0,5%, Tween20 0,05%, antibiotics mix

BUF03: Coating buffer with sodiumhydrogencarbonate 0,05M, sodium azide 0,01%, with pH9,6

BUF04: 2% BSA in PBS

BUF05: Casein buffer with NAOH 0,05M, EDTA 0,0015M, casein 2,5%

BUF06: 1% gelatin & 20% saccharose buffer diluted with H2O

BUF07: Sample dilution buffer with TBS, Tween20 0,05%, antibiotics mix

BUF08: Lyophilization buffer with PBS, BSA 2%, saccharose 20%, gentamycin 10mg/ml and antibiotics mix