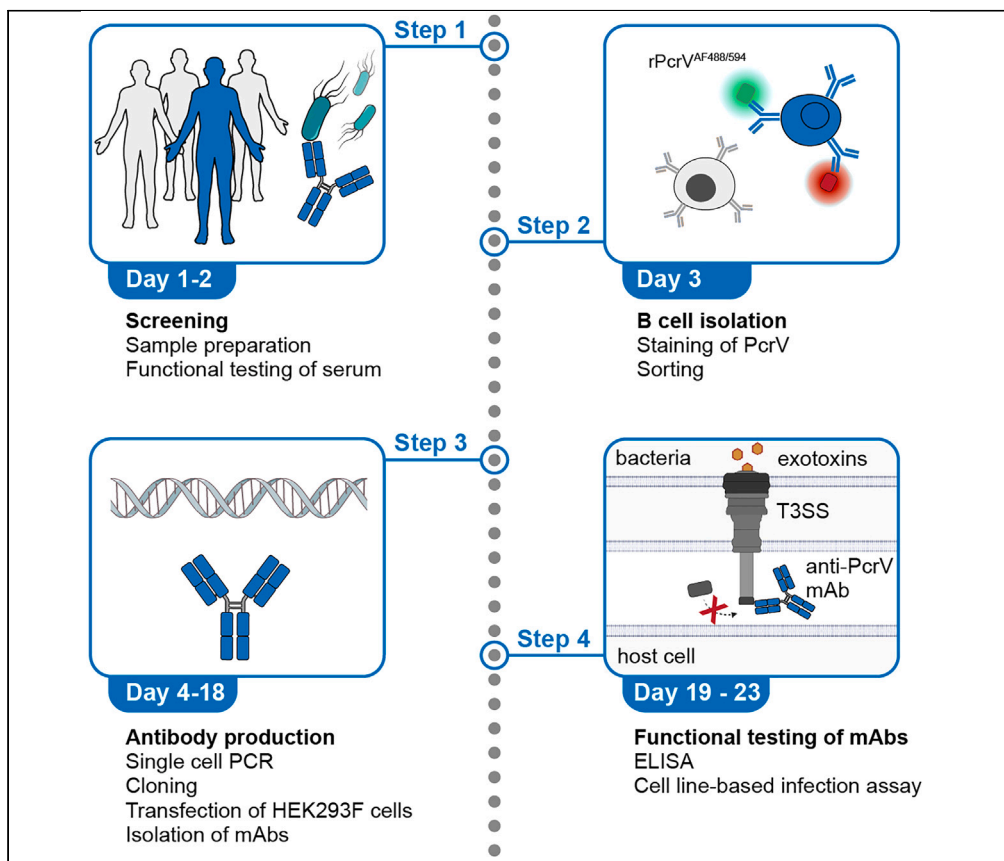


Protocol

Protocol for developing *Pseudomonas aeruginosa* type III secretion system-neutralizing monoclonal antibodies from human B cells



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Highlights

Functional screening
for type III secretion
system-neutralizing
antibodies in humans

Production of patient-
derived anti-PcrV
antibodies from
single B cells

In vitro
characterization of
anti-PcrV antibodies

Monoclonal antibodies (mAbs) targeting bacterial virulence factors may represent promising therapeutics in the fight against severe bacterial infections. Here, we present an approach for developing human-derived antibodies targeting the type III secretion system (T3SS) of *Pseudomonas aeruginosa* (PA) by neutralizing the function of the T3SS-tip protein PcrV. The protocol involves identifying individuals with protective antibodies, isolating PcrV-specific B cells from these individuals, and producing and testing anti-PcrV mAbs derived from single B cells.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol

Protocol for developing *Pseudomonas aeruginosa* type III secretion system-neutralizing monoclonal antibodies from human B cellsAlexandra Albus,^{1,2} Christoph Kreer,³ Florian Klein,^{3,4} Jan Rybníček,^{1,4} and Alexander Simonis^{1,2,4,5,6,*}¹Department I of Internal Medicine, Faculty of Medicine and University Hospital Cologne, University of Cologne, 50937 Cologne, Germany²Center for Molecular Medicine Cologne (CMMC), Faculty of Medicine and University Hospital Cologne, University of Cologne, 50931 Cologne, Germany³Laboratory of Experimental Immunology, Institute of Virology, Faculty of Medicine and University Hospital Cologne, University of Cologne, 50931 Cologne, Germany⁴German Center for Infection Research (DZIF), Partner Site Bonn-Cologne, 50937 Cologne, Germany⁵Technical contact⁶Lead contact*Correspondence: alexander.simonis@uk-koeln.de
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SUMMARY

Monoclonal antibodies (mAbs) targeting bacterial virulence factors may represent promising therapeutics in the fight against severe bacterial infections. Here, we present an approach for developing human-derived antibodies targeting the type III secretion system (T3SS) of *Pseudomonas aeruginosa* (PA) by neutralizing the function of the T3SS-tip protein PcrV. The protocol involves identifying individuals with protective antibodies, isolating PcrV-specific B cells from these individuals, and producing and testing anti-PcrV mAbs derived from single B cells.

For complete details on the use and execution of this protocol, please refer to Simonis et al.¹

BEFORE YOU BEGIN

This protocol details the methodology for developing human-derived mAbs that neutralize the type III T3SS function of PA. The T3SS is a critical virulence factor correlated with increased disease severity, functioning as a syringe-like structure that injects effector toxins into host cells, leading to cell lysis and tissue damage.² The PcrV protein is essential for this mechanism, forming the T3SS-needle tip complex required for the assembly and insertion of the PopB/D translocon into host cell membranes.³

Given the pivotal role of virulence factors in the pathogenesis of bacterial infections, investigating the human antibody repertoire against these factors and identifying neutralizing patient-derived antibodies presents a promising therapeutic strategy.⁴ This protocol facilitates the identification of highly T3SS-neutralizing antibodies from humans, offering potential new treatments for PA infections.

The presented workflow is based on the antibody isolation protocol from Gieselmann et al.⁵ but modified for the production of anti-PcrV mAbs and complemented with critical PA-specific assays, which can also be adapted for other T3SS proteins. Before initiating the study, it is necessary to recruit a cohort with individuals with prolonged or chronic PA infections and collect serum samples, peripheral blood mononuclear cells (PBMCs), and to produce purified PcrV protein.



Table 1. PcrV PCR master mix

Reagent	Amount per tube (μL)
Nuclease-free H ₂ O	16.38
5× Phusion GC Buffer	6.0
DMSO	0.9
dNTP mix (25 mM)	0.6
PcrV fwd primer (10 μM)	3.0
PcrV rev primer (forward) (10 μM)	3.0
Phusion High-Fidelity DNA Polymerase	0.12
Total	30

Institutional permissions

The use of human samples for laboratory experiments requires approved institutional review board (IRB) approval. Your institution may require additional and/or region-specific permissions, as well as restrictions with some materials/reagents. Therefore, do not start working before the necessary approvals have been obtained. Data shown in this protocol were obtained under the approval of the Institutional Review Board (IRB) of the University of Cologne, Germany (20-1287_1).

Pseudomonas aeruginosa is classified as biosafety level 2. Please note that you must fulfill all biosafety regulations before starting.

Preparation of serum samples and PBMCs

⌚ **Timing:** 120 min

⚠ **CRITICAL:** Antigen exposure is essential for the induction of a B cell response. Therefore, the study cohort should include individuals who have previously been infected or colonized with PA.

1. Serum preparation.

- Collect 4 – 9 mL peripheral blood in a 9 mL serum blood collection tube.
- After blood clotting, centrifuge the tube for 10 min at 3,000 × g at room temperature (RT = 18 – 24°C).
- Transfer the supernatant (serum) into 1.5 mL microcentrifuge tubes.
- Incubate the serum at 56°C for 30 min (e.g., in a dry block heater) to inactivate the complement.
- Centrifuge the tube for 1 min at 15,000 × g at RT and transfer supernatant into a new tube.
- Store the heat-inactivated serum at –80°C until further use.

Note: Preparation of small volume aliquots is recommended to avoid repeated freeze-thaw cycles of serum samples.

2. Preparation of PBMCs samples.

PBMCs samples are required for the isolation of PcrV-specific B cells.

Note: We recommend PBMCs sampling after identification of individuals in the screening assay (see section ‘[identification of individuals for single B cell sorting](#)’).

- Collect 150 – 400 mL of peripheral blood in 9 mL ethylenediaminetetraacetic acid (EDTA) collection tubes or a blood bag with citrate phosphate dextrose adenine solution (CPDA-1).

Note: The maximal volume of blood donation should be adapted to the body weight and health status of the donor. PBMC yield ranges between 0.5 – 3 × 10⁶ cells per mL blood.

Table 2. PcrV PCR program

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	2.5 min	1
Denaturation	95°C	30 s	35 cycles
Annealing	68°C	30 s	
Extension	72°C	60 s	
Final extension	72°C	10 min	1
Hold	4°C	forever	

For isolation of PcrV-specific B cells a minimum of 1×10^8 PBMCs should be used. Higher yields can be achieved by using leukapheresis.

- b. Dilute peripheral blood with phosphate buffered saline (PBS) containing 2 mM EDTA in a 1:1 ratio and transfer 30 mL per LeucoSEP tube. Prepare each LeucoSEP tube with 15 mL Ficoll beforehand. One LeucoSEP tube will be required for every 15 mL of peripheral blood.

Note: Instead of using LeucoSEP tubes, samples can be loaded directly on 15 mL Ficoll for gradient centrifugation.

- c. Centrifuge LeucoSEP tubes for 15 min at $800 \times g$ at 4°C. Perform this step with acceleration set to 9 and deceleration set to 0.

Note: After centrifugation, four distinct layers can be observed from top to bottom: plasma, PBMCs, Ficoll, and erythrocytes. The PBMC layer will appear as a thin whitish line just below the yellowish plasma layer at the top.

- d. Carefully transfer the PBMC layers of all LeucoSEP tubes to a new 50 mL tube and add ice-cold PBS containing 2 mM EDTA to a final volume of 45 mL. Use several new 50 mL tubes if needed.

Note: An additional incubation step with 0.83% ammonium chloride in PBS containing 2 mM EDTA for 5 min on ice can reduce contamination with RBCs and increase PBMC purity.

- e. Centrifuge the tube for 10 min at $400 \times g$ at 4°C. Perform this step with acceleration set to 9 and deceleration set to 9 (all subsequent centrifugation steps are performed with these settings).
- f. Discard the supernatant and repeat the washing step twice with ice-cold PBS containing 2 mM EDTA for 10 min at $200 \times g$ at 4°C.

Note: Centrifugation steps with $200 \times g$ are necessary to discard thrombocytes.

- g. Resuspend the cell pellet in 45 mL PBS containing 2 mM EDTA and determine the cell count.
- h. Centrifuge the tube for 10 min at $300 \times g$ at 4°C.
- i. Resuspend pellet in ice-cold freezing medium (Fetal calf serum (FCS) with 10% (v/v) dimethyl sulfoxide (DMSO); filtered with a 0.2 μ m filter) and transfer into a cryovial.

Note: We recommend $5 \times 10^7 - 1 \times 10^8$ cells/mL freezing medium.

- j. Store samples at -150°C until further use.

Note: For cryopreservation use a controlled rate cryo-freezer or a cryo-freezing container. We recommend labeling the cryovials prior to aliquoting the cells, as it is not advisable to keep the cells in cryopreservation medium for extended periods. Place the cryovials in a

freezing container and store them immediately at -80°C for 24 h. For long-term storage, transfer the vials to liquid nitrogen or at least -150°C .

Recombinant expression of PcrV

⌚ Timing: 5 days

3. Cloning of the *pcrV* gene into an pQE80 vector.
 - a. Isolate genomic DNA from a PA strain of interest using the DNeasy Blood & Tissue Kit according to the manufacturer's instructions ([Manual](#)).
 - b. Amplify the *pcrV* gene using Phusion High Fidelity Polymerase with the following primer pair:
Forward: TCACCATCACGGATCCGAAGTCAGAAACCTTAATG.
Reverse: TCAGCTAATTAAGCTTCTAGATCGCGCTGAGAATG.
A detailed PCR protocol is provided in [Tables 1](#) and [2](#).
 - c. Digest 1 μg of the expression vector (pQE80) with BamHI and HindIII (1 μL each = 20 units) for 15 min at 37°C .
 - d. Load complete sample on a 1.5% agarose gel and run at 120 V.
 - e. Cut the linearized vector band (approximately 4600 bp) from the gel and purify it using a gel extraction kit (e.g., QIAquick Gel Extraction Kit).
 - f. Purify the PCR product by using a PCR purification kit (e.g., QIAquick PCR Purification Kit) and clone it into the linearized expression vector with a 1:1 ratio with 200 ng using the In-Fusion HD EcoDry Cloning Kit according to the manufacturer's instructions ([Manual](#)).
 - g. Select clones on ampicillin-supplemented agar plates (ampicillin concentration 100 $\mu\text{g}/\text{mL}$).

Note: Selection of antibiotic is dependent on the expression vector.

4. Transformation and protein expression.
 - a. Grow colonies containing the recombinant vectors in 5 mL LB (lysogeny broth) liquid cultures supplemented with 100 $\mu\text{g}/\text{mL}$ ampicillin.
 - b. Isolate plasmids using the QIAprep Spin Miniprep Kit.

Note: Verify the accurate insertion of the *pcrV* gene by sequencing. For sequencing, use a pQE80 forward- (GCTTTGTGAGCGGATAACAATT) or reverse primer (ACTCCATCTGGA TTTGTTCA).

- c. Transform a plasmid with correct insert into competent BL21 *E. coli* cells by heat shock. Add 1 μg of the plasmid to a vial containing 50 μL BL21 *E. coli* cell suspension and incubate on ice for 30 min.
- d. Perform a heat shock by placing the vial in a 42°C water bath for 30 s, followed by a 2-min incubation on ice.
- e. Add 950 μL SOC (Super optimal broth with 20 mM glucose)-medium and incubate for shaking with 120 rpm for 1 h at 37°C .
- f. Plate 100 μL of the suspension onto an agar plate containing ampicillin (100 $\mu\text{g}/\text{mL}$) and incubate at 37°C overnight. Pick a single colony on the next day, transfer it into 10 mL LB media supplemented with 100 $\mu\text{g}/\text{mL}$ ampicillin and incubate overnight.
- g. Transfer the overnight culture into 240 mL fresh LB media.
- h. Grow the culture to an optical density (OD) at 600 nm of 0.5, then induce protein expression by adding isopropyl β -D-1-thiogalactopyranoside (IPTG) to a final concentration of 300 μM .
- i. Incubate the culture for an additional 3 h with shaking at 30°C with 120 rpm.
- j. Centrifuge bacterial suspension at $4000 \times g$ for 15 min.
- k. Lyse the bacterial pellet using B-PER Bacterial Protein Extraction Reagent according to the manufacturer's instructions ([Manual](#)).

- l. Equilibrate the bacterial lysate with 30 mM imidazole in a 1:1 ration (final concentration 15 mM imidazole).
- m. Load the lysate onto a 10 mL chromatography column with 2 mL bed volume loaded with 2 mL HisPur Ni-NTA resin and collect flowthrough. Repeat the step three times.
- n. Wash the columns eight times with 10 mL PBS containing 25 mM imidazole.
- o. Elute the His tag-labeled PcrV with 2 mL 250 mM imidazole.
- p. Exchange the buffer to PBS using Amicon Ultra-4 10 kDa spin filters by repetitive washing steps.
- q. Determine the purity of the recombinant PcrV by SDS-PAGE (e.g., by using a NuPAGE 4%–12% Bis-Tris midi gel) and staining of protein bands e.g., with InstantBlue Protein Stain.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-human CD20-Alexa Fluor 700 (clone 2H7) (1:100)	BD Biosciences	Cat.# 560631; RRID: AB_1727447
Anti-human IgG-PE (clone G18-145) (1:20)	BD Biosciences	Cat.# 560951; RRID:AB_10563761
Goat anti-human IgG	Biotrend	Cat.# 2040-01
Goat anti-human IgG-HRP (1:5,000)	SouthernBiotech	Cat.# 2040-05; RRID: AB_2795644
Immunoglobulin G	Sigma-Aldrich	Cat.# 56834-100MG
Bacterial and virus strains		
<i>E. coli</i> DH5 α	Thermo Fisher Scientific	Cat.#18263012
<i>E. coli</i> BL21 (DE3)	NEB	Cat.#C2527H
<i>Pseudomonas aeruginosa</i> PAO1	Bleves et al. ⁶	N/A
<i>Pseudomonas aeruginosa</i> PA14	Budzik et al. ⁷	N/A
Biological samples		
Human red blood cells	This study	N/A
PBMCs, serum, and IgGs of donors	This study	N/A
Chemicals, peptides, and recombinant proteins		
10 $\times$ PBS	Thermo Fisher Scientific	Cat.# AM9625
ABTS solution	Thermo Fisher Scientific	Cat.# 002024
Ampicillin sodium salt	Sigma-Aldrich	Cat.# A0166
BamHI-HF	NEB	Cat.#R3136
Bovine serum albumin (BSA)	Sigma-Aldrich	Cat.# A9418
BsiWI-HF	NEB	Cat.#R3553
Calf intestinal phosphatase	NEB	Cat.#M0525S
CutSmart buffer	NEB	Cat.#B6004S
DAPI	Thermo Fisher Scientific	Cat.#D1306
DMSO	Merck	Cat.#D2650
DNA loading dye	Thermo Fisher Scientific	Cat.#R0611
dNTP mix	Thermo Fisher Scientific	Cat.#R1122
DPBS	Thermo Fisher Scientific	Cat.# 14190250
DTT	Promega	Cat.#P1171
EcoRI-HF	NEB	Cat.#R3101
EDTA	Thermo Fisher Scientific	Cat.# AM9260G
ELISA coating buffer	BioLegend	Cat.# 421701
Ethanol	Carl Roth	Cat.# 9065.4
Fetal calf serum (FCS)	Thermo Fisher Scientific	Cat.# 10270-106
Ficoll-Paque Plus	Cytiva	Cat.# 17-1440-03
FreeStyle expression medium	Thermo Fisher Scientific	Cat.# 12338001
Gentamicin sulfate	Sigma-Aldrich	Cat.#G1914
Glycerol	Merck	Cat.#G5516
Glycine	Carl Roth	Cat.# 3187.3

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
HindIII-HF	NEB	Cat.#R3104
Imidazole	Sigma-Aldrich	Cat.#I202
InstantBlue protein stain	Expedeon	Cat.# ISB1L
IPTG (isopropyl β-d-1-thiogalactopyranoside)	Sigma-Aldrich	Cat.#I6758
KB extender (part of the Platinum Taq DNA polymerase)	Thermo Fisher Scientific	Cat.# 10966034
LB medium (Luria/Miller)	Carl Roth	Cat.#X968.2
MgCl ₂ 50 mM (part of the Platinum Taq DNA polymerase)	Thermo Fisher Scientific	Cat.# 10966034
Moxifloxacin-hydrochloride	Sigma-Aldrich	Cat.# SML1581
NEB 2.1 buffer	NEB	Cat.#B6002S
NP-40	Thermo Fisher Scientific	Cat.# 85124
Nuclease-free water	Thermo Fisher Scientific	Cat.# AM9937
Phusion high-fidelity GC buffer	Thermo Fisher Scientific	Cat.#F519L
Phusion high-fidelity DNA polymerase	Thermo Fisher Scientific	Cat.#F530L
Platinum Taq DNA polymerase	Thermo Fisher Scientific	Cat.# 10966034
Platinum Taq Green PCR Buffer (part of the Platinum Taq Green Hot Start)	Thermo Fisher Scientific	Cat.# 11966034
Platinum Taq Green Hot Start	Thermo Fisher Scientific	Cat.# 11966034
PEI (Polyethylenimine), 25 kDa	Sigma-Aldrich	Cat.# 408727
Protein G Sepharose 4 fast flow	GE Life Sciences	Cat.# 17061805
Q5 high GC enhancer (part of the Q5 high-fidelity DNA polymerase)	NEB	Cat.#M0491S
Q5 high-fidelity DNA polymerase	NEB	Cat.#M0491S
Q5 hot start high-fidelity DNA polymerase	NEB	Cat.#M0493L
Q5 PCR buffer (part of the Q5 high-fidelity DNA polymerase)	NEB	Cat.#M0491S
Random hexamer primer	Thermo Fisher Scientific	Cat.# SO142
Resazurin	Sigma-Aldrich	Cat.#R7017
RNaseOUT	Thermo Fisher Scientific	Cat.# 10777019
RNasin	Promega	Cat.#N2515
RPMI 1640	Thermo Fisher Scientific	Cat.# 11875093
Sall-HF	NEB	Cat.#R3138
Skim milk powder	Merck	Cat.# 70166
SOC medium	Thermo Fisher Scientific	Cat.# 15544034
SuperScript IV reverse transcriptase	Thermo Fisher Scientific	Cat.# 18090050
T4 DNA polymerase	NEB	Cat.#M0203L
Tris-(hydroxymethyl)-aminomethane	Carl Roth	Cat.# 4855.3
Trypsin-EDTA	Thermo Fisher Scientific	Cat.# 25300-096
Tween 20	Merck	Cat.# 817072
XhoI	NEB	Cat.#R0146

Critical commercial assays

Alexa Fluor 488 microscale protein labeling kit	Thermo Fisher Scientific	Cat.# A30006
Alexa Fluor 647 microscale protein labeling kit	Thermo Fisher Scientific	Cat.# A30009
Amicon Ultra-4 10 kDa spin filters	Millipore	Cat.# UFC901008
Amicon Ultra-4 30 kDa spin filters	Millipore	Cat.# UFC903008
B-PER bacterial protein extraction reagent	Thermo Fisher Scientific	Cat.# 90084
CD19 MicroBeads	Miltenyi Biotec	Cat.# 130-050-301
DNeasy blood & tissue kit	QIAGEN	Cat.# 69504
HisPur Ni-NTA resin	Thermo Fisher Scientific	Cat.# 88221
In-Fusion HD EcoDry cloning kit	Takara Bio	Cat.# 080318
LeucoSEP	Greiner	Cat.# 227290
LS columns	Miltenyi Biotec	Cat.# 130-042-401
NucleoSpin 96 PCR clean-up	MACHERY-NAGEL	Cat.# 740658.4
Nunc-Immuno 96 MicroWell	Merck	Cat.#M5785-1CS
NuPAGE 4%–12%, Bis-Tris	Thermo Fisher Scientific	Cat.# WG1402BOX
Poly-Prep chromatography columns	Bio-Rad	Cat.# 7311550

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
QIAquick gel extraction kit	QIAGEN	Cat.# 28704
QIAprep spin miniprep kit	QIAGEN	Cat.# 27104
QIAquick PCR purification kit	QIAGEN	Cat.# 28104
Experimental models: Cell lines		
A549	ATCC	Cat.# CCL-185; RRID:CVCL_0023
HEK293-6E	NRC	NRC file 11565
Oligonucleotides		
PcrV fwd (TCACCATCACGGATCCGAAGTCAGAAACCTTAATG)	This study	N/A
PcrV rev (TCAGCTAATTAAGCTTCTAGATCGCGCTGAGAATG)	This study	N/A
pQE80 forward primer (GCTTTGTGAGCGGATAACAATT)	This study	N/A
pQE80 reverse primer (ACTCCATCTGGATTGTTC)	This study	N/A
Random hexamer primer	Thermo Fisher Scientific	Cat.# SO142
Single-cell PCR primer 1 st PCR (oPR-IGHV, IGKV, IGLV and reverse primers)	Gieselmann et al., ⁵ Kreer et al. ⁸	N/A
Single-cell PCR primer 2 nd PCR (oPR-IGHV, IGKV, IGLV and reverse primers)	Gieselmann et al., ⁵ Kreer et al. ⁸	N/A
SLIC primer (SLIC_oPR_IGHV, SLIC_oPR_IGKV, SLIC_oPR_IGLV and reverse primers)	Gieselmann et al. ⁵	N/A
Recombinant DNA		
Human antibody expression vectors (IgG1, Igλ, Igκ)	Tiller et al. ⁹	N/A
pQE-80L	QIAGEN, NovoPro	Cat.#V010777
Software and algorithms		
Clustal Omega 1.2.3 (http://www.clustal.org/omega/)	Sievers et al., ¹⁰ Ye et al. ¹¹	RRID: SCR_001591
IgBLAST 1.13.0 (https://ftp.ncbi.nih.gov/blast/executables/igblast/release/LATEST/)	Ye et al. ¹¹	RRID: SCR_002873

MATERIALS AND EQUIPMENT

MACS buffer		
Reagent	Final concentration	Amount
EDTA (0.5 M)	2 mM	2 mL
BSA	1% (w/v)	5 g
PBS	N/A	Up to 500 mL
Store at 4°C for up to 3 months.		

FACS buffer		
Reagent	Final concentration	Amount
FCS	2% (v/v)	10 mL
EDTA (0.5 M)	2 mM	2 mL
BSA	1% (w/v)	5 g
PBS	N/A	Up to 500 mL
Store at 4°C for up to 3 months.		

Blocking buffer (ELISA)		
Reagent	Final concentration	Amount
BSA	2.5% (w/v)	2.5 g
Skim milk	2.5% (w/v)	2.5 g
Tween 20	0.05% [v/v]	50 μL
PBS	N/A	Up to 100 mL
Store at 4°C for up to 1 week.		

Blocking buffer (Cell sorting)

Reagent	Final concentration	Amount
FCS	10% (v/v)	5 mL
BSA	5% (w/v)	2.5 g
PBS	N/A	Up to 50 mL

Store at 4°C for up to 3 months.

Staining buffer (Cell sorting)

Reagent	Final concentration	Amount per 100 μ L
Anti-Human CD20-AF700	1:100	1 μ L
IgG-PE	1:20	5 μ L
DAPI (Live/Dead)	3 μ M	105 ng
PcrV ^{AF488}	10 μ g/mL	1 μ g
PcrV ^{AF647}	10 μ g/mL	1 μ g
FACS Buffer	N/A	Up to 100 μ L

Sorting buffer

Reagent	Final concentration	Amount per 1 mL
Nuclease-free H ₂ O	N/A	775 μ L
RNasin (40 U/ μ L)	2 U/ μ L	50 μ L
RNaseOut (40 U/L)	1 U/L	25 μ L
PBS (10 $\times$)	0.5 $\times$	50 μ L
DTT (100 mM)	1 mM	100 μ L

Use directly or store at -80°C for up to 3 months.

STEP-BY-STEP METHOD DETAILS

Identification of individuals for single B cell sorting

⌚ Timing: 2 days

To identify individuals with highly neutralizing anti-PcrV antibodies, a combination of PcrV-binding ELISA and functional assays should be used. Screening can be conducted using either serum samples or isolated IgG. If serum samples are utilized, it is recommended to measure the IgG concentrations to normalize the results based on the IgG content of each sample. The effects measured in functional assays should be specifically sensitive to T3SS-dependent activities such as T3SS-mediated cytotoxicity. Due to its sensitivity and specificity, we recommend the described hemolysis assay. However, other functional assays that rely on T3SS functionality may also be suitable.

- PcrV-binding ELISA.
 - Dilute PcrV protein in ELISA coating buffer (e.g., from BioLegend) to a final concentration of 2 μ g/mL.
 - Add 100 μ L of the diluted PcrV solution to each well of a MaxiSorp ELISA 96-well plate.
 - Seal the plate and incubate overnight at 4°C.
 - Wash the ELISA plate three times with PBST (PBS + 0.05% [v/v] Tween 20) and discard the washing buffer.
 - Incubate the plate with blocking buffer (2.5% [w/v] bovine serum albumin [BSA] and 2.5% [w/v] skim milk in PBST) for at least 1 h at room temperature (RT) with constant agitation.
 - Dilute patient sera (from step 1) in a 1:4 serial dilution on a separate non-binding microplate (96-well) in blocking buffer. We recommend starting with a serum dilution of 1:32, progressing to a final dilution of 1:32,768.

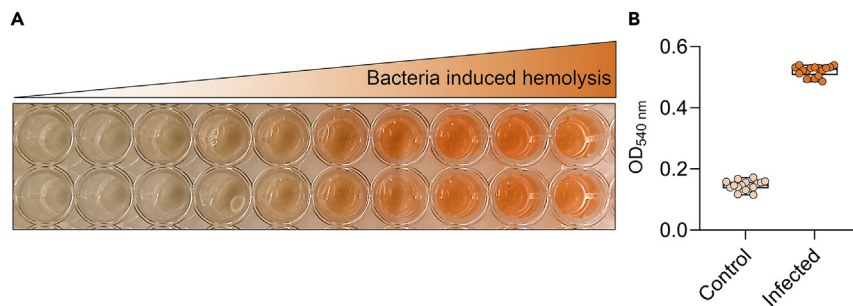


Figure 1. Quantification of PA-induced hemolysis

(A) Supernatant of RBCs with increasing bacteria-induced hemolysis shown in duplicates.
(B) Hemolysis was quantified by measuring the optical density (OD) at 540 nm from uninfected RBCs (control) and RBCs infected at an MOI of 1. Boxplots indicate the median, the upper and lower quartile, and the minimum and maximum values. Shown data points represent the technical mean of an independent experiment.

- g. Discard the blocking buffer from the ELISA plate and wash the ELISA plate three times with PBST, discard the washing buffer.
 - h. Transfer 100 μ L of the diluted sera from the titration plate to the ELISA plate.
 - i. Incubate for 2 h at RT with constant agitation (80 rpm).
 - j. Wash the ELISA plate three times with PBST and discard the washing buffer.
 - k. Add 100 μ L goat anti-human IgG-HRP secondary antibody in blocking buffer (1:5,000) to each well.
 - l. Incubate for 1 h at RT at 80 rpm.
 - m. Wash the ELISA plate four times with PBST and discard the washing buffer.
 - n. Add 100 μ L of ABTS substrate solution to each well and incubate at RT.
 - o. Measure the absorbance at 415 nm after 15 min using a microplate reader.
2. Functional testing of serum using hemolysis assay.

△ CRITICAL: A Class 2 safety cabinet is required for working with PA. All steps involving open handling with PA should be performed under a Class II safety cabinet. Ensure all biosafety level 2 handling requirements are met. For the calculation of T3SS neutralization, including a positive control (infection of RBCs without serum) and a negative control (incubation of RBCs with PBS without bacteria) is necessary.

- a. Grow PA strain PA14 overnight at 37°C with shaking at 180 rpm.
- b. Adjust the OD_{600 nm} of the overnight culture to 0.2 in fresh LB media and incubate at 180 rpm at 37°C until the OD_{600 nm} reaches 1.
- c. During growth of bacteria prepare human blood samples:
 - i. Collect 9 mL of peripheral blood in a 9 mL EDTA collection tube.
 - ii. Transfer the blood into a 50 mL tube and add PBS to a final volume of 45 mL.
 - iii. Centrifuge at 200 $\times$ g at RT for 10 min with deceleration set to 0.
 - iv. Discard the supernatant carefully and resuspend the blood in PBS to a total volume of 45 mL. Repeat the washing step two more times.
 - v. Adjust the red blood cell (RBC) count to 5 $\times$ 10⁸ cells/mL in PBS.

Note: Washed blood can be stored for up to 5 days at 4°C in RPMI containing 10% FCS. Before reuse, the cell culture medium must be removed by washing. Centrifuge the tube at 200 $\times$ g at RT for 10 min. Discard the supernatant, then fill the tube with PBS up to 45 mL and repeat the centrifugation. Perform the PBS washing step one more time.

- d. After reaching an OD_{600 nm} of 1, centrifuge bacteria suspension at 4,000 g at RT for 10 min.
- e. Resuspend bacteria pellet in PBS to reach a concentration of 5 $\times$ 10⁸ bacteria/mL.

Note: At an OD_{600 nm} of 1 PA concentration is approximately 1.5×10^8 bacteria/mL. Correlation of OD_{600 nm} and bacterial count should be determined by plating and count of colony forming units (CFUs) before experiments are performed.

- f. Transfer 125 μ L bacteria suspension per well into a round-bottom microplate and add serum to reach a final serum dilution between 1:100–1:800. Use PBS at the corresponding dilutions as control.
- g. Seal the plate and preincubate for 20 min at RT with constant agitation (80 rpm).
- h. After 20 min, add 125 μ L of the erythrocyte suspension to each well and reseal the plate.
- i. Centrifuge the plate at $1,000 \times g$ at RT for 5 min.
- j. Remove the sealing tape and incubate the plate in an incubator at 37°C with 5% CO₂ for 2 h.
- k. Resuspend each well, reseal, and centrifuge at $1,500 \times g$ at RT for 10 min.
- l. Carefully transfer 100 μ L of the supernatant from each well to a flat-bottom 96-well microplate and measure the OD at 540 nm to quantify bacteria-induced hemolysis (Figure 1).

Note: Avoiding air bubbles and resuspension of RBC is crucial, as these can cause false positive measurements.

Isolation of PcrV-specific B cells

⌚ Timing: 1 day

Isolation of PcrV-specific B cells is pivotal for the protocol. As PcrV-specific B cells are expected under 0.1% of all IgG⁺ B cells, we recommend using at least 1×10^8 PBMCs for the experiments. The workflow follows the protocol from Gieselmann et al.⁵ with some minor modifications and has been adapted to recombinant PcrV-protein as a sorting bait.

⚠ **CRITICAL:** Stringent selection of the target population, using controls (e.g., PBMCs from a healthy donor), careful adjustments of the cell sorter as well as sterile/RNA and DNA-free preparations of the sorting buffer and PCR plate are crucial to obtain reliable results.

3. Fluorophore-labeling of PcrV.
 - a. Label two batches of PcrV (each 100 μ g) (from step 'recombinant expression of PcrV') with two different fluorophores separately. We recommend using commercially available labeling kits (e.g., Alexa Fluor Microscale Protein Labeling Kit) according to the manufacturer's instructions and the settings of the cell sorter (Manual). To avoid spectral overlaps, we recommend using Alexa Fluor 488 Microscale Protein Labeling Kit for one batch and Alexa Fluor 647 Microscale Protein Labeling Kit from Thermo Fisher Scientific for the other batch.

Note: Labeled PcrV can be stored at 4°C protected from light. Given the uncertain long-term stability of labeled PcrV, it is advisable to use it within a few days after labeling.

4. B cell enrichment and staining.
 - a. Thaw PBMCs of a donor with a high PcrV titer and evidence of neutralizing effects in the screening assay (see 'identification of individuals for single B cell sorting'), transfer to a 50 mL tube and fill the tube with ice-cold MACS buffer to 45 mL final volume.
 - b. Centrifuge at $300 \times g$ at 4°C for 10 min and discard the supernatant.
 - c. Repeat the washing step, resuspend the pellet in 10 mL ice-cold MACS buffer and count the cells.
 - d. Centrifuge at $300 \times g$ at 4°C for 10 min and resuspend the pellet with 80 μ L MACS buffer per 1×10^7 cells.
 - e. Add 20 μ L magnetic CD19-microbeads per 1×10^7 cells and incubate the cells for 15 min at 4°C.

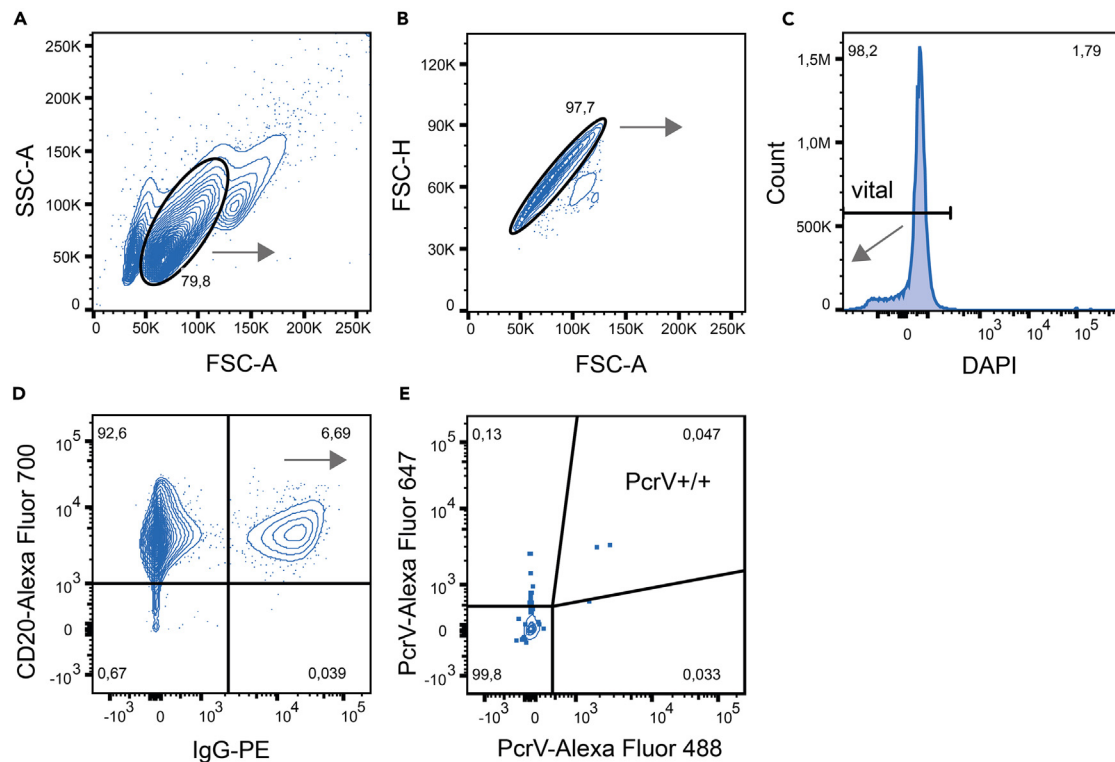


Figure 2. Gating strategy for the isolation of PcrV-specific B cells

- (A) The gate represents the lymphocyte population.
 (B) The gated population from A is plotted, with settings adjusted to exclude doublets.
 (C) The gated population from B is further analyzed for DAPI staining, and only DAPI-negative cells are selected for subsequent gating.
 (D) The DAPI-negative cells from C are analyzed for IgG (x-axis) and CD20 (y-axis) expression. Double-positive cells (CD20⁺ IgG⁺) in the upper right quadrant are selected for further analysis.
 (E) CD20⁺ IgG⁺ cells which are double-positive for PcrV-binding (upper right quadrant) will be sorted in a single-cell manner.

- f. Add 20 mL MACS buffer and centrifuge at $300 \times g$ at 4°C for 10 min.
- g. Resuspend the pellet in 0.5 mL MACS buffer per 1×10^8 cells.
- h. Place a cell strainer on LS columns for positive cell selection and place the columns in a corresponding magnetic cell separator. Wash columns with 5 mL MACS buffer.
- i. Load the cell suspension onto the prepared column, collect flow through (if needed) and wash the column three times with 5 mL MACS buffer.
- j. Discard the cell strainer and fill the column with 5 mL fresh MACS buffer. Detach the column from the magnetic cell separator and use the syringe plunger to elute the CD19⁺ cells.

Note: Use the plunger carefully to elute the cells from the column. Slow down when foam appears.

- k. Centrifuge the flow through at $300 \times g$ at 4°C for 10 min in a 50 mL tube. Discard the supernatant.
- l. Resuspend CD19⁺ cells in 5 mL fresh MACS buffer and count the cells.

Note: The expected amount of CD19⁺ cells are around 10% of the initial PBMC number.

- m. Centrifuge the cells at $300 \times g$ at 4°C for 10 min. Discard the supernatant.
- n. Resuspend CD19⁺ cells in blocking buffer (PBS containing 10% FCS and 5% BSA) for 30 min at 4°C.

Table 3. 1st RT master mix

Reagent	Amount per well (μL)	Amount per plate (μL)
Nuclease-free H ₂ O	5.6	616
Random hexamer primers (200 ng/μL)	0.75	82.5
NP40 (10% (vol/vol))	0.5	55
RNaseOut (40 U/μL)	0.15	16.5
Total	7	770

- o. Add 20 mL FACS (Fluorescence Activated Cell Sorting) buffer and centrifuge at 300 × g at 4°C for 10 min. Discard the supernatant.
- p. Add staining buffer to the cells (100 μL/1 × 10⁷ cells), resuspend and incubate for 20 min at 4°C protected from light.
- q. Wash the cells with 45 mL FACS buffer and resuspend the cells in FACS buffer at a final concentration of 2.5 × 10⁶ /mL.
- r. Transfer the stained cells through a 40 μm cell strainer into a tube compatible with the respective cell sorter.
5. Single Cell Sorting.
 - a. Before sorting, prepare PCR plates with sorting buffer.
 - i. Add 4 μL sorting buffer per well into a PCR clean 96-well PCR plate.
 - ii. Seal the plate using PCR-suitable foil. Store the sorting plates on ice for immediate use or at –80°C for long-term storage if prepared in advance.
 - b. Set sorter specifications for different lasers and perform compensation for each fluorophore.

Note: We recommend using the CD19⁺ fraction and staining these cells with CD3 antibodies for each fluorophore. Additionally, use B cells from healthy donors without a PcrV titer as a negative control and for defining the gate of the PcrV-positive cells.

- c. Sort PcrV-specific B cells in a single-cell manner (Figure 2).

Note: To minimize the risk of contamination, it is recommended to perform sorting with a cell sorter within a laminar flow hood.

- d. Seal the plate immediately after sorting and store at –80°C until further use.

Single B cell PCR and production of anti-PcrV mAbs

⌚ **Timing:** 14 days

The production of mAbs is a critical component of this project. Given the sensitivity of single B cell PCR, it is imperative to handle this step with extreme care to prevent contamination or sample mix-up. Maintaining an organized working and storage structure is essential. B cell PCR, B cell receptor cloning, and antibody production are performed according to the antibody isolation protocol from Gieselmann et al.⁵

⚠ **CRITICAL:** To prepare for single B cell work, clean all surfaces and pipettes with 70% ethanol followed by RNase away, and then expose to UV irradiation. Additionally, wear disinfected sleeves and a face mask to further minimize contamination. We strongly recommend to perform all work under a PCR hood.

6. Reverse Transcription.
 - a. Prepare the 1st (Table 3) and the 2nd RT master mix (Table 4), excluding addition of SuperScript IV.

Table 4. 2nd RT master mix

Reagent	Amount per well (μL)	Amount per plate (μL)
Nuclease-free H ₂ O	2.05	205
SuperScript IV RT Buffer (5×)	3	300
dNTP mix (25 mM)	0.5	50
DTT (100 mM)	1	100
RNasin (40 U/μL)	0.1	11
RNaseOut (40 U/μL)	0.1	11
Intermediate sum	6.75	675
SuperScript IV (200 U/μL)	0.25	25
Total	7	700

- b. Thaw the sorting plate on ice and centrifuge at 600 × g at 4°C for 1 min.
- c. Transfer 1st RT master mix into a sterile reservoir, carefully add 7 μL per well into the sorting plate and resuspend several times.

Note: Use of multichannel pipettes is recommended.

⚠ **CRITICAL:** Use new tips for each well to avoid cross-contamination between wells.

- d. Seal the plate with PCR foil and incubate at 65°C for 2.5 min.
- e. Place the plate on ice for at least 2 min.
- f. Add SuperScript IV to the 2nd RT master mix and transfer 7 μL per well, then seal the plate.
- g. Centrifuge at 600 × g at 4°C for 1 min.
- h. Place the plate in a thermocycler and run the reverse transcription program (Table 5).
- i. Add 16 μL of nuclease-free water per well.
- j. Store the plate at –80°C until further use or proceed with 1st PCR.
7. Amplification of heavy and light chain genes.
 - a. Prepare the 1st PCR master mix for amplifying heavy and light chain genes (Tables 6 and 7).
 - b. Add 19 μL of master mix per well in a 96-well PCR plate.
 - c. Thaw the RT plate from step 6.
 - d. Resuspend and transfer 3.8 μL per well from the RT plate to the PCR plate.
 - e. Seal the plate and centrifuge at 600 × g at 4°C for 1 min.
 - f. Place the plate in a thermocycler and run the 1st PCR protocol (Table 8).
 - g. Proceed with h.) or store the plate at 4°C for short-term storage or at –80°C for long-term storage.
 - h. Prepare separate 2nd PCR master mix (Tables 9 and 10).
 - i. Add 19 μL of 2nd PCR master mix per well in a 96-well PCR plate.
 - j. Resuspend and transfer 3 μL per well from the 1st to 2nd PCR plates (1 × heavy, 1 × kappa, 1 × lambda).
 - k. Seal the plate and centrifuge at 600 × g at 4°C for 1 min.
 - l. Place the plate in a thermocycler and run the 2nd PCR protocol (Table 11).
 - m. Proceed to step 3 or store the plate at 4°C for short-term storage or at –80°C for long-term storage.

Table 5. Reverse transcription program

Steps	Temperature	Time	Cycles
1	42°C	10 min	1
2	25°C	10 min	1
3	50°C	10 min	1
4	94°C	5 min	1
Hold	4°C	forever	

Table 6. 1st PCR master mix

Reagent	Amount per well (μL)
Nuclease-free H ₂ O	14.68
Platinum Taq PCR buffer (10×)	2.05
KB Extender (6%)	1.23
MgCl ₂ (50 mM)	0.61
dNTP mix (25 mM)	0.16
oPR_1st_fwd primer mix (50 μM)	0.09
oPR_1st_IgG_rev primer mix (50 μM)	0.09
Platinum Taq DNA Polymerase	0.09
Total	19.0

8. Verification and analysis of heavy and light chain genes.
 - a. Load 4 μL/sample on a 2% agarose gel and run at 120 V.

Note: Expected product lengths for heavy chains will be around 500 bp and 450 bp for light chains.

- b. Evaluate amplicons by sequencing. Use the respective chain-specific reverse primer that was used for the second PCR.
 - c. Analyze sequencing results using tools such as NIH IgBlast. Sequences that are in-frame, without stop codons, and contain the full variable region should be considered productive.
9. Amplification of heavy and kappa or lambda chains using SLIC-Primer.
 - a. Prepare separate master mixes for the amplification of heavy and kappa or lambda chains (Tables 12 and 13).
 - b. Add 46 μL of the SLIC master mix per well in a 96-well PCR plate.
 - c. Use the PCR plate of the 1st PCR (from step 7), thaw if necessary.
 - d. Resuspend and transfer 1 μL per well per chain from the first PCR plate to the SLIC PCR plate.
 - e. Seal the plate and centrifuge at 600 × g at 4°C for 1 min.
 - f. Run the SLIC PCR protocol in a thermocycler (Table 14).
 - g. Load 4 μL/sample on a 2% agarose gel and run at 120 V.

Note: Expected product lengths for heavy chains will be around 600 bp and 550 bp for light chains.

- h. Proceed to step 5 or store the plate at 4°C for short-term storage or at –80°C for long-term storage.
10. Linearization of expression vector.
 - a. Prepare digestion for vector linearization (Table 15).
 - b. Heat digest at 37°C for 16 h and inactivate enzymes at 65°C for 20 min after digest.
 - c. Add 0.5 μL of calf intestinal phosphatase (CIP) per reaction and incubate at 37°C for 30 min.
 - d. Load complete sample on a 2% agarose gel and run at 120 V.
 - e. Cut the linearized plasmid using a clean scalpel for each plasmid to avoid cross-contamination.
 - f. Purify plasmids using a plasmid preparation kit.

Table 7. Primer 1st PCR

oPR_1st_fwd ^{5,8}	oPR_1st_IgG_rev ^{5,8}
oPR-IGHV-1_fw 1–15	Cg RT
oPR-IGKV fw 1–8	3' Cκ 543
oPR-IGLV 1–15_fw	3' Cλ

Table 8. 1st PCR program

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	2 min	1
Denaturation	94°C	30 s	50 cycles
Annealing	57°C	30 s	
Extension	72°C	55 s	
Final extension	72°C	6 min	1
Hold	4°C	forever	

- g. Proceed to step 11 or store the plate at 4°C for short-term storage or at –80°C for long-term storage.
11. Sequence and ligation independent cloning (SLIC).
 - a. Ligate the SLIC product with 8 ng/μL of linearized vector (Table 16). The molar ratio should be 1:6 to 1:2.
 - b. Incubate at 24°C for 15 min using a thermocycler. Place on ice for 10 min afterward.
 - c. Thaw competent *E. coli* DH5α on ice.

Note: Instead, commercially available vials, *E. coli* DH5α can be made competent by using calcium chloride.

- d. Add 4 μL of the SLIC reaction to the competent *E. coli* and incubate for at least 30 min.
- e. Incubate the reaction mix for 45 s at 42°C. Use a water-bath or a thermocycler.
- f. Place the vial on ice after heat-shock immediately.
- g. Add 50 μL SOC medium to each ligation mix.
- h. Incubate at 37°C and 200 rpm for 1 h.
- i. Plate ligation mixes on ampicillin-supplemented agar plates (ampicillin concentration 100 μg/mL) plates and incubate overnight at 37°C.

Note: Colonies may be visible after 16 h. Proceed to step 7 or store plates with colonies at 4°C for up to two weeks.

12. Colony PCR and sequencing.
 - a. Prepare Colony PCR master mixes for each chain type according to the number of colonies and transfer to PCR tubes (Table 17). Additionally, prepare 15 mL tubes with 5 mL LB-medium supplemented with ampicillin (100 μg/mL).
 - b. Pick a colony and resuspend it in the LB + ampicillin media. Use the same tip to resuspend in the designated PCR tube.
 - c. Run the protocol for the Colony PCR (Table 18).
 - d. Load 4 μL/sample on a 2% agarose gel and run at 120 V.

Table 9. 2nd PCR master mix

Reagent	Amount per well (μL)
Nuclease-free H ₂ O	14.68
Platinum Taq Green PCR buffer (10×)	2.05
KB Extender (6%)	1.23
MgCl ₂ (50 mM)	0.61
dNTP mix (25 mM)	0.16
5' primer mix (50 μM)	0.09
3' primer (50 μM)	0.09
Platinum Taq DNA polymerase	0.09
Total	19.0

Table 10. Primer 2nd PCR

IgG1 heavy chain ^{5,8}	Kappa chain ^{5,8}	Lambda chain ^{5,8}
oPR-IGHV-1_fw 1–15	oPR-IGKV-1_fw 1–8	oPR-IGLV-1_fw 1–15
3' IgG (internal) (rev)	3' Cκ 494 (rev)	3' XhoI Cλ (rev)

Note: Expected product lengths for heavy chains will be around 680 bp and 630 bp for light chains.

- e. Incubate LB-media of colonies with correct insert size at 37°C and 180 rpm overnight.
- f. Centrifuge at 4,000 × g at RT for 10 min.
- g. Isolate plasmids using a Plasmid Preparation Kit (e.g., QIAprep Spin Miniprep Kit).
- h. Verify correct insertion by sequencing using the 5' Ab-sense primer.⁵
- i. Plasmids can be stored at 4°C for short-term or at –20°C for long-term storage.

Note: We recommend preparing glycerol stocks of *E. coli* containing plasmids with the correct insertion for long-term storage and plasmid preparation. Mix glycerol and LB medium in a 1:1 ratio. Then, combine 600 μL of the overnight culture with 600 μL of the LB-glycerol mixture in a labeled tube, and store the stock at –80°C.

13. Recombinant expression of antibodies.
 - a. Grow HEK293E cells using Erlenmeyer culture flask in shaking incubator at 37°C at 120 rpm and 8% CO₂.
 - b. For transfection adjust a cell concentration of 0.8–1.2 × 10⁶ cells/mL.
 - c. Prepare transfection mix according to [Table 19](#).

△ CRITICAL: First, add the heavy and light chain plasmids to PBS. Immediately after adding PEI (Polyethylenimine), vortex for 15 s.

- d. Incubate for 10 min at RT and gently add the mixture to cells under constant agitation.
- e. Incubate the cells for 7 d at 37°C, 8% CO₂ and 120 rpm.
- f. After 7 d, centrifuge the cells at 3,800 × g at RT for 10 min.
- g. Transfer the supernatant into 50 mL tubes.

Note: The supernatant can be stored for up to two weeks at 4°C.

- h. Add Protein G Sepharose to the supernatant.

Note: We recommend at least 4 μL Protein G Sepharose per mL of supernatant. The IgG concentration in the supernatant can be measured using a human IgG Fc ELISA to determine the appropriate amount of Protein G resin. Protein G Sepharose has a binding capacity of 20 mg of human IgG per mL of resin.

- i. Incubate for 2 h at RT or at 4°C overnight under constant agitation.

Table 11. 2nd PCR program

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	2 min	1
Denaturation	94°C	30 s	50 cycles
Annealing	57°C	30 s	
Extension	72°C	45 s	
Final extension	72°C	6 min	1
Hold	4°C	forever	

Table 12. SLIC PCR master mix

Reagent	Amount per well (μL)
Nuclease-free H ₂ O	24.1
Q5 PCR buffer (5×)	10
dNTP mix (25 mM)	0.4
5' primer mix (50 μM)	0.5
3' primer (50 μM)	0.5
Q5 High GC Enhancer (5×)	10
Q5 high-fidelity DNA polymerase	0.5
Total	46

- j. Centrifuge at 600 g at 4°C for 5 min (with deceleration set to 0).
- k. Discard the supernatant and resuspend the Protein G Sepharose with 5 mL PBS per 1 mL Protein G Sepharose.
- l. Prepare Poly-Prep chromatography columns by adding 10 mL with 70% ethanol followed by 10 mL PBS.
- m. Add the resuspended Protein G Sepharose to the columns and wash three times with 10 mL PBS.
- n. Elute IgG directly into tubes containing 500 μL of 1 M Tris buffer (pH 8) by adding 2.5 mL of 0.1 M Glycine buffer (pH 2.7) on the columns.
- o. Prepare Amicon Ultra-4 30 kDa spin filters using 2 mL PBS and centrifuge at 3,800 × g at 4°C for 5 min.
- p. Discard flowthrough and apply eluate with antibody concentrations greater than 20 μg/mL to spin filters.

Note: Antibody concentration can be measured using UV/Vis spectroscopy, using a 1:10 dilution of Tris in glycine buffer as the blank.

- q. Exchange buffer by repeatedly adding up to 4 mL PBS and centrifuging at 3,800 × g at 4°C for 10 min.

Note: Dilute Tris/Glycine at least by 1:200.

- r. After buffer exchange, filter the mAbs using a 0.2 μm sterile filter to prevent contamination and remove aggregates.

Note: Purified antibodies can be stored at 4°C for up to 6 months.

Functional validation of anti-PcrV mAbs in an A549-based cytotoxicity assay

⌚ Timing: 3 days

Before proceeding with functional testing, the affinity of PcrV mAbs should be assessed using a PcrV-binding ELISA (see step 1 'PcrV-binding ELISA'). Additionally, we recommend conducting functional testing with the described hemolysis assay to evaluate neutralizing activity.

Table 13. Primer SLIC PCR

IgG1 heavy chain ⁵	Kappa chain ⁵	Lambda chain ⁵
SLIC_oPR_IGHV 1–15	SLIC_oPR_IGKV 1–8	SLIC_oPR_IGLV 1–15
SLIC_oPR_IgG_HC_rev	SLIC_oPR_IGKV_rev	SLIC_oPR_IGLV_rev

Table 14. SLIC PCR program

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	35 cycles
Annealing	72°C	40 s	
Final extension	72°C	2 min	1
Hold	4°C	forever	

14. A549 cytotoxicity assay.

- Culture A549 cells in RPMI medium supplemented with 10% FCS (RF) in a T75 flask, maintaining the flask in a humidified incubator at 37°C with 5% CO₂.
- Seed 20,000 cells/well in 100 µL RF medium in a 96-well cell culture plate.
- Inoculate PAO1 into 5 mL LB media and incubate at 37°C with 180 rpm shaking overnight.
- Prepare a fresh PAO1 culture by transferring 1 mL of the overnight culture into 9 mL of fresh LB and incubating at 37°C with 180 rpm shaking until reaching an OD_{600 nm} of 1.
- Prepare a serial dilution of anti-PcrV mAbs starting at a final concentration of 50 µg/mL to 5 ng/mL on a 96-well non-binding titration plate. Dilute mAbs in RF medium with a final volume of 10 µL.

Note: Use an unspecific IgG antibody as well as gentamicin (20 µg/mL) as controls.

- Use PAO1 culture to prepare a bacterial suspension with 222,000 bacteria/mL in RF medium to reach a final multiplicity of infection (MOI) of 0.5.
- Add to each mAb 90 µL of the bacterial suspension.
- Seal the plate and preincubate at room temperature for 20 min with shaking at 80 rpm.
- Discard the cell culture medium from the A549 cells and replace it with the PAO1/mAb mixture (from step e).
- Incubate the cells for 3 h in the humidified incubator at 37°C with 5% CO₂.
- Wash the cells by discarding the media and replacing it with fresh RF medium.
- Discard the medium and replace it with 100 µL/well RF medium containing 20 µg/mL gentamicin and 10 µg/mL moxifloxacin.
- Incubate the plate in the humidified incubator at 37°C with 5% CO₂ for 16 h.
- Add 10 µL/well of resazurin (0.25 mg/mL).
- Incubate the plate in the humidified incubator at 37°C with 5% CO₂.
- Measure fluorescent signal using 530–570 nm excitation wavelength and 585 – 590 nm emission wavelength after 120 min ([Figure 3](#)).

EXPECTED OUTCOMES

With the present protocol, we present a workflow for the identification of individuals with highly neutralizing antibodies targeting PcrV of the T3SS of PA. Successful completion of the protocol should enable the generation of human-derived neutralizing PcrV mAbs.

Table 15. Restriction digests

IgG1 heavy chain	Kappa chain	Lambda chain
10 µg vector	10 µg vector	10 µg vector
2.5 µL EcoRI-HF (50 U)	2.5 µL EcoRI-HF (50 U)	2.5 µL EcoRI-HF (50 U)
2.5 µL SalI-HF (50 U)	2.5 µL BsiWI-HF (50 U)	2.5 µL XhoI (50 U)
5 µL CutSmart Buffer	5 µL CutSmart Buffer	5 µL CutSmart Buffer
40 µL nuclease-free H ₂ O	30 µL nuclease-free H ₂ O	30 µL nuclease-free H ₂ O

Table 16. SLIC reaction master mix

Reagent	Amount/well (μL)
Nuclease-free water	to 4
Linearized vector	Final concentration 8 ng/μL
NEB Buffer 2.1 (10×)	1
T4 DNA Polymerase (3,000 u/mL)	0.2
Total	4

Expected percentage of individuals with highly neutralizing antibodies

In a predominantly chronically infected cystic fibrosis cohort, approximately 30% of individuals exhibited serum with neutralizing effects, reducing PA-induced cytotoxicity by more than 50% at a 1:800 dilution.¹ Notably, none of the healthy control group ($n = 51$) reached this threshold at either a 1:800 or 1:200 serum dilution.¹ This finding underscores the importance of careful cohort selection and should be considered prior to initiating the protocol.

Expected clonality of PcrV-specific B cells

To analyze PcrV-specific B cells, we recommend examining the VH/VJ gene distribution and assessing both germline and CDRH3 identities among the individual B cells. By defining clonal groups based on identical VH/VJ genes and CDRH3 identities of $\geq 75\%$, we identified several clusters. These clusters can be utilized for the selection of monoclonal antibody (mAb) candidates (Figure 4).

Expected yield of anti-PcrV mAbs

The yield of anti-PcrV monoclonal antibodies varied among different clones. At a cell concentration of $0.8\text{--}1.2 \times 10^6$ cells/mL, the average antibody yield is approximately 20 μg/mL.

Expected neutralizing capacity of anti-PcrV mAbs

Through this workflow, we identified individuals with highly neutralizing serum, followed by the recombinant expression of anti-PcrV mAbs. The neutralizing capabilities varied among different clones and donors, with a minimum IC50 of 56 ng/mL (Figure 5).

LIMITATIONS

The identification of individuals for the described workflow is based on the functional characterization of T3SS activity and the detection of anti-PcrV mAbs in their serum. It is important to consider that T3SS inhibition may also be attributable to antibodies binding to other T3SS proteins, rather than exclusively to PcrV.

TROUBLESHOOTING

Problem 1

PcrV titer measurement in serum shows high background (related to step 1).

Table 17. Colony PCR reaction master mix

Reagent	Amount/well (μL)
Nuclease-free water	17.43
Platinum Tag Green PCR Buffer (10×)	2.20
KB Extender (6%)	1.28
MgCl ₂ (50 mM)	0.65
dNTP Mix (25 mM)	0.2
5' Ab-sense (50 μM)	0.08
3' primer (50 μM)	0.08
Platinum Taq DNA Polymerase	0.08
Total	22.00

Table 18. Colony PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	5 min	1
Denaturation	94°C	30 s	28 cycles
Annealing	55°C	30 s	
Extension	72°C	1 min	
Final extension	72°C	5 min	1
Hold	4°C	forever	

Potential solution

- Increase blocking time up to 3 h at RT or block overnight at 4°C and add 2 additional washing steps to each (step 1).
- Verify PcrV purity by SDS-PAGE.

Problem 2

Low number of PcrV-specific B cells (related to step 5).

Potential solution

- Low recovery or viability of isolated PBMCs can be mitigated by isolating PBMCs promptly after collection and ensuring meticulous cryopreservation techniques.
- Percentage of PcrV-specific B cells is low (<0.1% of all CD20⁺ IgG⁺), therefore, it is highly recommended to obtain a minimum of 1×10^8 PBMCs. The total number of PBMCs can be significantly increased by utilizing leukapheresis instead of conventional blood drawing.
- Test sufficient fluorophore-labeling of PcrV by spectrophotometry.
- Re-evaluate the individual's PcrV titer. If necessary, select or screen for an individual with a high PcrV titer.

Problem 3

Low antibody yields (related to step 13).

Potential solution

- Production of antibodies decreases with increasing passages of HEK293-6E cells. Obtain a new batch of HEK293-6E cells and repeat the transfection process.

Problem 4

Produced human-derived mAbs do not bind PcrV (related to step 13).

Potential solution

- Ensure proper blocking before staining to avoid excessive PcrV positivity.
- Compensation and accurate gating before sorting are crucial (Figure 2).
- Use a negative control (B cells from a donor without titer) to accurately distinguish specific PcrV binding.

Table 19. Transfection

Reagent	Amount/1 mL culture volume
DPBS	45 μ L
Heavy chain plasmid	0.5 μ g
Light chain plasmid	0.5 μ g
PEI (0.45 mg/mL)	3.4 μ L

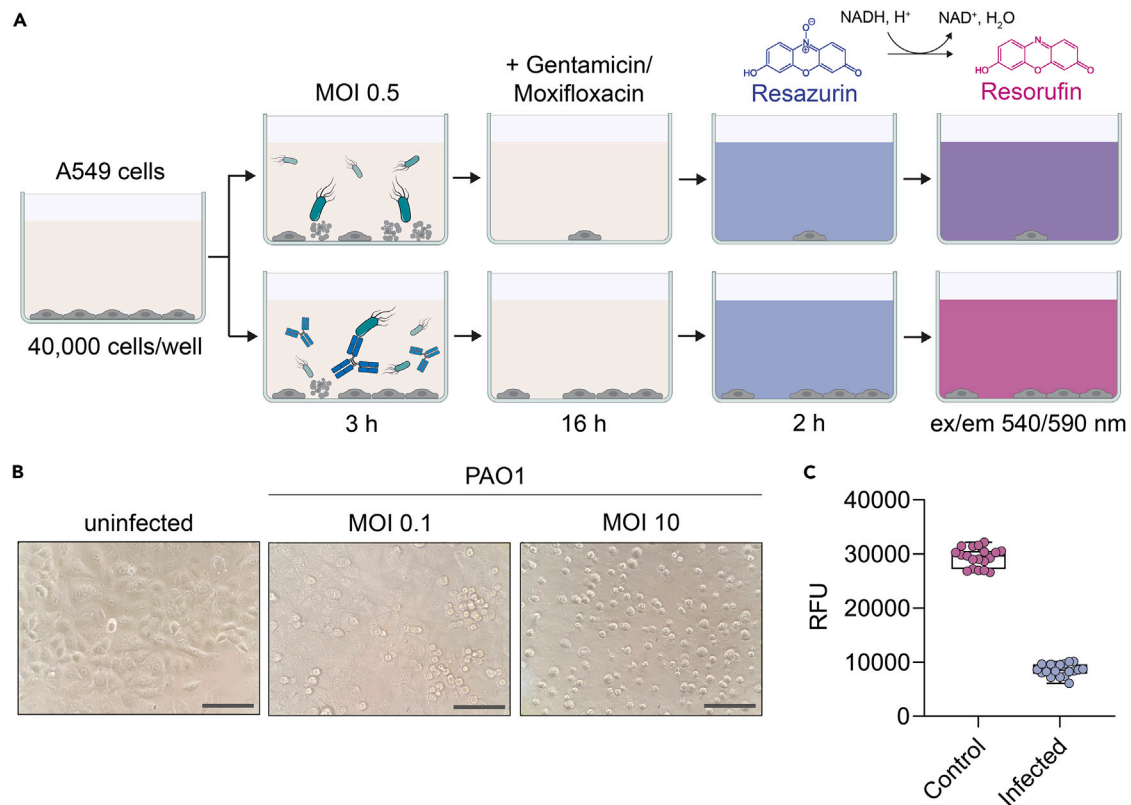


Figure 3. A549-based cytotoxicity assay

(A) Illustration of the experimental setup, which is described in this protocol (step 14).

(B) A549 cells were infected with the PA wild-type strain PAO1 at a MOI of 0.1 and 10 for 3 h. Uninfected cells were used as a control. Changes in cell morphology, indicative of bacteria-induced cell death, were documented using bright-field microscopy. Scale bar indicate 20 μ m.

(C) Relative fluorescence units (RFU) were measured after adding resazurin to uninfected cells (control) and cells infected with a MOI of 0.5 for 3 h. Boxplots indicate the median, the upper and lower quartile, and the minimum and maximum values. Shown data points represent the technical mean of an independent experiment.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Alexander Simonis (alexander.simonis@uk-koeln.de).

Technical contact

For detailed technical questions, please reach out to the technical contact, Alexander Simonis (alexander.simonis@uk-koeln.de).

Materials availability

All materials except for generated mAbs and patient material is available as mentioned in the materials section. Primers have been ordered by local provider. Plasmids used in this study have been ordered from Addgene.

Data and code availability

All data reported in this protocol will be shared by the [lead contact](#) upon request, and this protocol does not report original code.

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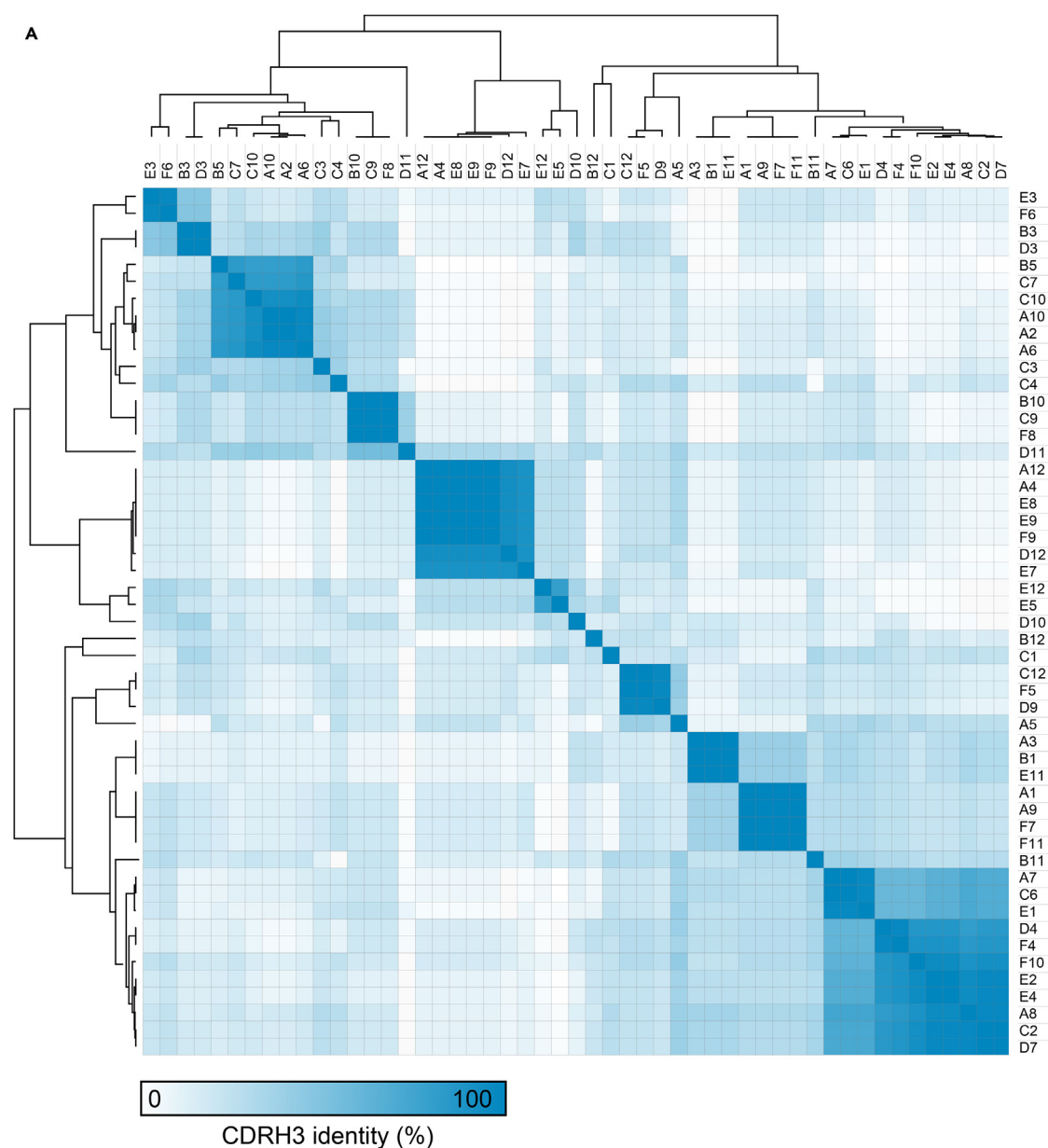


Figure 4. CDRH3 Identity of Single PcrV-Specific B Cells

(A) CDRH3 identity of single PcrV-specific B cells ($n = 51$) from a single donor was analyzed. Sequences were hierarchically clustered using Clustal Omega (ClustalO), and a similarity matrix was generated based on Pearson correlation.

of Cologne, University of Cologne; and the German Center for Infection Research (DZIF) (to F.K., TTU 09.719 and DZIF TTU 02.806 to J.R.). Graphical illustrations were created with BioRender.com.

AUTHOR CONTRIBUTIONS

Conceptualization, F.K., J.R., and A.S.; methodology, A.A., C.K., and A.S.; investigation, A.A.; formal analysis, A.A. and A.S.; writing – original draft, A.A. and A.S.; writing – review and editing, C.K., F.K., J.R., and A.S.; supervision, A.S.; funding acquisition, F.K., J.R., and A.S.

DECLARATION OF INTERESTS

A patent application for a set of highly neutralizing antibodies developed using the described workflow has been filed by the University of Cologne, with A.S., C.K., F.K., and J.R. listed as inventors.

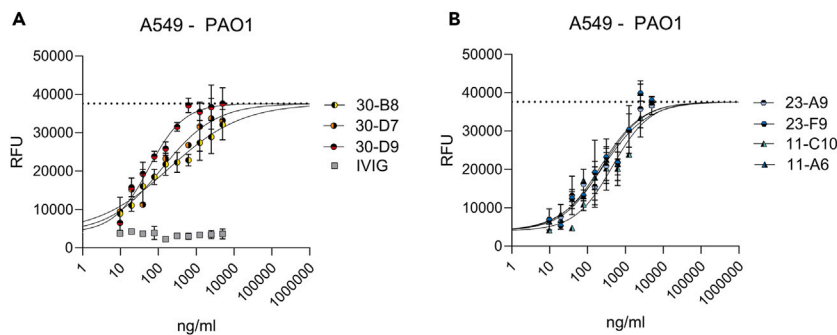


Figure 5. A549-based cytotoxicity assay

(A and B) A549 cells were infected with PAO1 for 150 min with a MOI of 0.5 in presence of patient-derived monoclonal anti-PcrV antibodies (at a concentration ranging from 5 μ g/mL to 10 ng/mL) ($n = 7$). As control, cells were left uninfected (dotted line) or were treated with polyclonal IgG (intravenous IgG = IVIG). Relative fluorescence units (RFU) were measured after adding resazurin. Shown data points represent the technical mean of an independent experiment. Error bars indicate standard deviation of the mean.

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