Test-Luciferase-Based Cytotoxicity Assay with bispecific antibodies

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We use this protocol and it's working

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ABSTRACT

The purpose of this assay is to determine the cytotoxic potential of bispecific antibodies.

This protocol describes the preparation and analysis of an in vitro assay with human target cells, human effector cells and bispecific antibodies (bsAb), which may be secreted into cell culture supernatants, found in mouse plasma/serum or prepared as a chromatographically-purified protein.

GUIDELINES

If not specified differently all procedures are performed under sterile conditions at RT.
As primary human material is used as effector cells, all work needs to meet biosafety level 2 requirements.

MATERIALS

Materials:
10 µL pipette Manual, single channel, operating volume 0.5 – 10 µL
100 µL pipette Manual, single channel, operating volume 10 – 100 µL
1000 µL pipette Manual, single channel, operating volume 100 – 1000 µL
20 µL pipette Manual, single channel, operating volume 2 – 20 µl
200 µL pipette Manual, single channel, operating volume 20 – 200 µL
Centrifuge Swing-out rotor and adapters for vessels, with plate rotors incl. fixed angle rotor for high speed centrifugation,
Dispenser/ Multipette® Direct displacement pipette, e.g. Modell Multipette® Xstream or Multipette® M4
Flow cytometer high throughput screening (HTS), e.g. FACSCanto II, FACSCelesta
Incubator CO2 concentration range 0 – 20 %, relative humidity up to 95 %, temperature range (metrical) 3 – 55 °C
Integra Voyager Electrically adjustable tips, 8- or 12-channel

Laboratory glass flask Max. volume 500 mL
Laminar flow class II biological safety cabinet, incl. H14 HEPA filter
Luminescence plate reader for microtiter plates
Measuring cylinder max. volume 500 mL
pH electrode
pH meter
Pipettor e.g. accu-jet® pro
Roller Mixer
Test tube shaker
Ultra-pure water system Purelab Chorus 1
Vacuum device
384-well plate
96-deepwell plate 1000 µL
96-deepwell plate 2000 µL
96-well luminescence plates, transparent
96-well luminescence plates, white
96-well microtiter plate, flat bottom
96-well microtiter plate, round bottom
AeraSeal™ film
Aluminum foil
Aspirating pipette
Cell culture flask 75 cm^2
Cell culture flask 175 cm^2
Eppendorf reaction tubes, 0.5 mL amber
Eppendorf reaction tubes, 1.5 mL
Eppendorf reaction tubes, 1.5 mL amber
Eppendorf reaction tubes, 2.0 mL
FACS tubes, 5 mL
Multistepper pipette tips 1 mL
Multistepper pipette tips 2.5 mL
Multistepper pipette tips 5 mL
Parafilm® M
Pipette tip rack
Single-use reservoir
Suspension culture flask 250 mL
Suspension culture flask 650 mL
Syringe filter, 0.22 µM pore size, surfactant-free, cellulose acetate membrane, single-use, sterile
Syringe 10 mL
Syringe 2 mL
Transfer pipettes 5, 10, 25, 50 mL
Tube 15 mL
Tube 50 mL

Reagents:

D-Luciferin Potassium Salt
RPMI 1640 Medium
Accutase®
anti-EpCAM muIgG2a
APC-conjugated AffiniPure F(ab')2 Fragment Goat Anti-Human IgG + IGM (H+L)
APC-conjugated AffiniPure F(ab')2 Fragment Goat Anti-Mouse IgG + IGM (H+L)
Aqua B. Braun
BD™ Stabilizing Fixative
CTL-Wash™ Supplement 10x
DNAse I
DPBS 1x
EDTA
eFluor 506
FBS Superior
HEPES
HMFG1
Human albumin
Hydrochloric acid (HCl)
IMAB027 Anti-human CLDN6, cross-reactive with mouse, not conjugated
IMAB027 Alexa Fluor 647 Anti-human CLDN6, cross-reactive with mouse, conjugated, for FACS
IMAB206 Anti-human CLDN6, cross-reactive with mouse, not conjugated
IMAB362 Anti-human CLDN18.2, cross-reactive with mouse, not conjugated, for FACS
IMAB362 Alexa Fluor 647 Anti-human CLDN18.2, cross-reactive with mouse, conjugated, for FACS
NG2 (9.2.27) Anti-human CSPG4, not conjugated
Pan T Cell Isolation Kit, human, Miltenyi Biotec, Cat. no 130-096- 535
Penicillin-Streptomycin
Triton™ X 100
Ultra-pure water
α-CD20-APC Anti-human CD20, APC fluorochrome, for FACS, Beckman Coulter, A21693
α-CD25-PE Anti-human CD25, PE fluorochrome, for FACS, BD™ Biosciences, Cat. no 555432
α-CD4-FITC Anti-human CD4, FITC fluorochrome, for FACS, BD™ Biosciences, Cat. no 555346
α-CD69- PerCP Anti-human CD69, PerCP tandem fluorochrome, for FACS, BD™ Biosciences, Cat. no 340548
α-CD8-APC anti-human CD8, APC fluorochrome, for FACS, BD™ Biosciences, Cat. no 345775
α-EGFR AF647 anti-EGFR, clone 528, AlexaFluor647 fluorochrome, for FACS, Santa Cruz Biotechnology, Cat. no sc-120 AF647

SAFETY WARNINGS

All samples must be treated as potentially infectious since primary human material is used.
Appropriate precautions need to be observed and protective clothing must be worn.

Preparation

1 Required Supplies

It is necessary to obtain stock solutions and sample material before beginning an experiment.

1.1 Stock Solutions

BD Monolight™ Luciferin
Final concentration: 12 mg/mL in water, pH 6.0 – 6.3
Due to possible racemization of D-luciferin to L-luciferin at pH above 6.3, this salt dissolution process needs to be performed quickly. 250 mg BD Monolight™ D-luciferin potassium salt is dissolved in 20 mL Aqua B. Braun at RT, protected from light. Therefore, 10 mL water is pipetted with a 10 mL transfer pipette into the BD™ glass bottle. The luciferin salt is dissolved by forward-reverse pipetting several times. With the same pipette, the dissolved luciferin is transferred into an amber or aluminum-wrapped 50 mL tube. Using a fresh 10 mL pipette, the glass bottle is rinsed with 10 mL water which is then transferred into the 50 mL tube. This step is repeated one to two times for collecting all luciferin salt in the original bottle, after which the remaining solution is collected with a 100 µL pipette. The 50 mL tube is then closed and inverted several times, followed by pH adjustment to 6.0 – 6.3 using a pH electrode, for which approx. 2 µL of 6 M HCl solution is needed. After pH adjustment, the final volume of the luciferin stock solution of 20.833 mL is reached by topping up with water using a 1000 µL pipette. The stock solution is aspirated with a 20 mL syringe and filtered through a syringe filter (0.2 µm) into a fresh 50 mL tube. Finally, aliquots of 1 mL are prepared in 1.5 mL amber reaction tubes and stored at −20 ± 5 °C.
Storage: −20 ± 5 °C, amber 1.5 mL reaction tubes.
Shelf life: 12 months.

Fixative Solution for FACS
Final concentration: 1x in water
To prepare the FACS fixative solution, one part BD™ Stabilizing Fixative is diluted with two

parts water in volume. 100 µL fixative is used per sample. The required total volume, tube size and pipettes are based on the total sample count. The solution is thoroughly mixed by manual inversion or vortexing prior to use.
Storage: 2 – 8 °C in polystyrene tubes.
Shelf life: 1 day.

Antibody staining solution (FAC staining buffer)
Final concentration: 5 % FBS, 5 mM EDTA in 1xDPBS 30 mL from a 500 mL DPBS bottle is discarded. Afterwards 25 mL FBS using a 25 mL transfer pipette and 5 mL 0.5 M EDTA using a 5 mL transfer pipette are added. The flask is tightly closed and manually inverted multiple times to obtain a homogenous solution.
Storage: 2 – 8 °C
Shelf life: 3 months.

Assay medium
Final concentration: 10 % FBS, 0.5 % P/S in RPMI 1640 medium
Under sterile conditions, 52.5 mL RPMI 1640 medium is removed from the medium flask using a 50 mL transfer pipette. 50 mL heat-inactivated FBS and 2.5 mL P/S are transferred into the medium flask using a 25 mL/50 mL transfer pipette or a 5 mL transfer pipette, respectively. The flask is tightly closed and inverted several times to obtain a homogenous solution. A sterility check is then performed according to SOP-020-009 “Ansetzen von Medien und Zusätzen für die Zellkultur“ and documented in FOR-020-009A. The heat inactivation of FBS destroys components of the complement system a part of the IgG effector function hence not heat inactivated assay medium has to be used for the investigation of the cytotoxic potential of IgG antibodies.
Storage: 2 – 8 °C
Shelf life: 1 month.

Triton™ X-100 lysis solution (for Lmax)
Final concentration: 20 % (v/v) in water.
10 mL Triton™ X-100 are transferred with a dispenser pipette into a 100 mL glass bottle, topped up with 40 mL ultra-pure water and mixed by vortexing.
Storage: RT
Shelf life: 12 months.

Luciferin solution
Final concentration: 3 mg/mL luciferin, 150 mM HEPES buffer To prepare a master mix for the working solution, the following components are sequentially mixed into a brown or aluminum-wrapped tube of appropriate size (usually 15 mL). The final volume is calculated according to the number of sample-containing wells. Sufficient extra volume also needs to be calculated. Example for a 96-well plate incl. extra volume, if 20 µL luciferin solution per well is used:
• 1440 µL Aqua B. Braun
• 360 µL 1 M HEPES buffer
• 600 µL Luciferin stock solution

The working solution is prepared at RT and is ready to use.
Long-term storage is not possible.

1.2 Sample Material

Samples for investigation can be chromatographically-purified bispecific antibodies (bsAb) in buffer or antibody-containing CCSN from producer cell lines (e.g. HEK-293, CHO-K1, K-562 cells) or mouse serum and plasma.
To determine the concentration Gyros ELISA, ForteBio BLItz, Nano Drop or Western Blot can be performed. Sample material needs to be stored at 2 – 8 °C.

2 Preparation of Effector Cells

The total number of human effector cells required is determined during planning of the experiment. The effectors can be used after being freshly isolated from buffy coats, pre-cultured or directly after thawing.
If fresh cells are used, an adequate number of buffy coat bags needs to be ordered at least 48 h in advance from the blood bank. On average, one buffy coat bag yields approx. $1x10^8$ PBMC. The buffy coats are stored at RT. No particular HLA-type or CMV status is required.

2.1 Isolation of effector cells

Human PBMC are isolated according to Ficoll gradient method. If purified T cells are required, they are isolated from bulk PBMCs via MACS separation. All effector cell counts need to be determined.

2.2 Thawing of effector cells

Prior to thawing, thawing medium need to be prepared, for which 10x CTL-wash is diluted 1:10 with RPMI 1640 medium in an appropriate tube. 2 µL DNAse I solution (1 mg/mL) is added per milliliter medium, resulting in 2 µg/mL DNAse I in the thawing medium. 9 mL thawing medium is needed per cryo-tube; the latter can be pooled for thawing if more than one. The cryo-tube/tubes are thawed in a 37 °C water bath. The cell suspension is aspirated with a 2 mL or 5 mL transfer pipette and added dropwise to the thawing medium. The tube is inverted und centrifuged at 300 xg, 8 min, RT. Finally, the cell count and viability are determined .The viability must be >80 %, below this the assay is not performed.

2.3 Determination of T-cell populations after isolation

The percentage of $CD4^+$ as well as $CD8^+$ T-cells in a PBMC population, the T-cell purity after MACS separation or the T-cell activation status is determined via FACS analysis.
For staining, $5x10^5$ cells per sample tube (pelleted at 460 xg, 5 min, RT program) are resuspended in FACS staining buffer at a 50 µL final volume containing the following antibodies:

- 3.0 µL α-CD4-FITC (ID043)

- 1.5 µL α-CD8-APC (ID044)
- 1.5 µL α-CD25-PE (ID047)
- 5.0 µL α-CD69-PerCP (ID117)
- 0.125 µL eFluor 506
- 38.875 µL staining buffer

A master mix is prepared if multiple samples are to be analyzed.
Since the negative control cells are stained only with the live/dead dye eFluor 506 without all other antibodies, the FACS antibody volume is replaced with staining buffer (11 µL per sample). All samples are vortexed for 3 s at moderate speed (level 4) and then incubated for 30 min at 2 – 8 °C in the dark.
Afterwards, 2 mL DPBS is distributed to all samples with a suitable transfer pipette and vortexed shortly. The sample tubes are then centrifuged, the DPBS supernatant discarded and the washing step repeated once more. With an appropriate pipette or dispenser, 100 µL fixative solution is added to each tube and the pellet dislodged/broken by quick vortexing. The sample tubes are then sealed e.g. with Parafilm® M and stored protected from light at 2 – 8 °C for max. three days.
The FACS sample acquisition is performed.
All gates must be adapted to the individual PBMC sample; an unchangeable template is therefore not applicable. Exclusion criteria for effectors with respect to the CD4+/CD8+ percentage or their activation status do not apply due to the need for FACS analysis, which is performed mostly after the assays themselves. The purity of T-cell populations after MACS isolation usually reaches >95 %; otherwise, the assay eventually needs to be repeated.

2.4 Determination of T-cell populations after thawing cryopreserved cells

For staining, $5x10^5$ cells per sample tube (pelleted at 460 xg, 5 min) are resuspended in FACS staining buffer at 50 µL final volume containing the following antibodies:
• 2.0 µL α-CD3-Pacific Blue (ID027)
• 1.5 µL α-CD56-PE-Cy7 (ID031)
• 3.0 µL α-CD4-FITC (ID043)
• 1.5 µL α-CD8-APC (ID044)
• 1.5 µL α-CD25-FITC (ID047)
• 0.5 µL α-CD19-FITC (ID095)
• 5.0 µL α-CD69-PerCP (ID117)
• 0.125 µL eFluor 506
• 34.88 µL FACS staining buffer

A master mix is prepared if multiple samples are to be analyzed. This panel differs from the freshly-isolated PBMC, as cryopreserved PBMC may also be used for other applications which require more detailed phenotyping. Thawed effectors are preferred to fresh cells, as the cell populations of each donor are already well characterized. In addition, the EC50 value are determined in a standard cytotoxicity assay3 after freezing and thawing.
s, for each assay, a specific donor can be chosen. FACS analysis for phenotyping is performed only once for each donor (=PBMC-bank). Further aliquots for use in assays are not analyzed again.

2.5 Cultivation of effector cells

PBMC

The isolated PBMC are kept light-protected in a closed sterile 50 mL tube at a cell density of 6 – 7x10^6 cells/mL in max. 30 mL assay medium at RT. The next day, the cells are centrifuged at 300 xg, 8 min, the supernatant removed with an aspiration pipette and the pellet resuspended in 1 mL assay medium by forward-reverse pipetting with a 1000 µL pipette. If the PBMC were cultivated in multiple tubes, they can be pooled after this step. The volume is then topped up to 50 mL with assay medium and the cell count and viability are determined. The viability must be >80 %, below which the assay is not performed.

T cells

Purified T cells are transferred into a cell culture flask with an appropriate pipette and the cell density is adjusted to 5x10^6 cells/mL with assay medium. The flask is incubated overnight at 37 °C with 5 % CO2, saturated humidity. The next day, T cells are transferred with a suitable pipette into a 50 mL tube. The flask is rinsed with fresh assay medium, which is also transferred into the 50 mL tube and centrifuged . The resulting supernatant is removed with an aspiration pipette and the pellet re-suspended in 1 mL assay medium with a 1000 µL pipette. The suspension is then topped up to 50 mL with assay medium, and the cell count and viability determined according. The viability must be >80 %, below which the assay is not performed.

2.6 Adjustment of effector cells

Effector cells are re-suspended in assay medium for the desired E:T ratio. Effector-cell suspension volumes of 40 µL and 10 µL are required for 96-well plates and 384-well plates, respectively, as shown in the table below. When analyzing samples from mouse i.e. plasma/serum (5 µL), the total cell suspension volume needs to be adapted appropriately for 96-well plates only. For 384-well plates the volumes and cell counts are not changed, the mouse samples are just added on top to the wells. The following table shows the counts for effector cells depending on the common E:T ratios:

Plate format	E:T	Effector#/well	Effector#/mL	Cell suspension/well [µL]
96-well	1:1	1x10^4	2.5x10^5	40
	3:1	3x10^4	7.5x10^5	
	5:1	5x10^4	1.25x10^6	
	10:1	1x10^5	2.5x10^6	
	30:1	3x10^5	7.5x10^6	
384-well	20:1	4x10^4	4x10^6	10

Table shows the counts for effector cells depending on the common E:T ratios

Different E:T ratios or volumes can be used equally. The adjusted effector cell suspension is stored in a 50 mL tube upon use at the same day at RT.

3 Preparation of Target Cells

The target cells are to be in their exponential growth phase for the assay. The required cell number is defined in the experimental plan and cells are expanded adequately prior to the assay day. The last passage takes place one or two days before.

The table below shows target molecules and corresponding target as well as control cells:

Target molecule	Target cells	Control cells
CLDN18.2	NUGC-4-3a_hCLDN18-2_luc_gfp_1F5	MDA-MB-231_luc_tom
CLDN6	PA-1-SC12_luc, OV-90-SC12_luc	MDA-MB-231_luc_tom
EGFR	NUGC-4-3a_hCLDN18-2_luc_gfp_1F5	Raji_hCLDN6_gfp_luc_1D6
EpCAM	OV-90-SC12_luc	Raji_hCLDN6_gfp_luc_1D6

3.1 Harvest of target cells (for 175 cm2 cell culture flask)

The culture medium is removed by aspiration and ~10 mL DPBS is then carefully added to the flask with an appropriate transfer pipette, avoiding disturbing the cell layer. The DPBS is distributed across the adherent cell layer by gently rocking the flask by hand, followed by fluid aspiration. For cell detachment, e.g. 4 mL Accutase® is added to the flask, gently handled as before and the cells placed in the incubator. The detachment process is monitored by microscopy. As soon as the majority of cells is detached, e.g. 6 mL DPBS or assay medium is added with a 10 mL transfer pipette, the cells transferred into a 15 mL tube and centrifuged at RT, 300 xg for 4 min. The resulting supernatant is discarded and the pellet is re-suspended in 1 mL followed by cell counting. The viability must be >80 %, otherwise the assay is not performed.

Harvest of target cells (for 650 mL cell culture flask)

The cell suspension is transferred into a 50 mL tube with an appropriate pipette and centrifuged at 300 xg, 4 min at RT. The resulting supernatant is discarded and the pellet is resuspended in 1 mL followed by cell counting. The viability must be >80 %, otherwise the assay is not performed.

3.2 Antigen expression check

Staining of target cells (S1 material) can be performed under non-sterile conditions outside the biosafety cabinet. $5x10^5$ cells are added to each 5 mL FACS sample tube, centrifuged at 460 xg, 5 min. and the supernatant decanted. 50 µL staining buffer including all antibodies and live/dead dye is added to the cell pellet with an appropriate pipette or dispenser. All samples are vortexed for 3 s at level 4 (moderate speed) and incubated in the dark at 2 – 8 °C for 30 min.

Sample list for staining with unconjugated primary antibodies:

Sample#	Primary Ab	Secondary Ab	eFluor 506 (0.125 µL/50 µL)
1. unstained	-	-	+
2. secondary Ab control	-	+	+
3. expression check	+	+	+
4. isotype control	+ (isotype)	+	+

Sample list for staining with fluorochrome-conjugated primary antibodies:

Sample#	Primary Ab	eFluor 506 (0.125 µL/50 µL)
1. unstained	N/A	+
2. expression check	+	+
3. isotype control	+ (isotype control)	+

Examples for frequently used antibodies for staining of tumor antigens:

Primary Ab	Ab ID	Final concentration [mg/mL]	Incubation time [min]	Tumor antigen	Secondary Ab (1:200)	Incubation time [min]
IMAB362-AF647	ID094	5	30	CLDN18.2	N/A	N/A
IMAB027-AF647	ID093	2.5	30	CLDN6	N/A	N/A
HMFG1	ID150	10	30	MUC-1	Anti-mouse APC	10
NG2 (9.2.27)	ID019	10	30	CSPG4	Anti-mouse APC	10
Anti-EpCAM	ID141	3	30	EpCAM	Anti-mouse APC	10
Anti-EGFR-AF647	ID144	10	30	EGFR	N/A	N/A

For the washing step, 2 mL DPBS is added to all samples, centrifuged at 460 xg for 5 min. and the DPBS supernatant is decanted. If a secondary antibody is required, add 50 µL staining buffer incl. secondary antibody. The tubes are vortexed for 3 s on level 4 (moderate speed) and incubated in the dark at 2 – 8 °C for 30 min. After this, 2 mL DPBS again is added to all samples, centrifuged at 460 xg for 5 min. and the is decanted. The tubes are vortexed for 3 s on level 4 (moderate speed) and the washing procedure is repeated once more.
Finally, 1x BD™ fixative solution, e.g. 100 µL, is added to each tube and vortexed (3 s, level 4 at moderate speed). The samples can be stored sealed for max. three days at 2 – 8 °C, protected from light. If the primary antibody is conjugated to a fluorescent dye, the washing

step is also performed twice. The FACS acquisition is performed. Data analysis is done by FlowJo.

3.3 Adjustment of target cells

$1x10^4$ targets cells/96-well and $2x10^3$ target cells/384-well plates, respectively, are needed. 40 µL/10 µL target-cell suspension are prepared. The total volume is calculated according to the total count of assay wells on all assay plates. Analyzing mouse-derived samples i.e. plasma/serum (5 µL per well) requires the total volume of cell suspension to be adapted appropriately.

Examples for cell counts and extra volumes for multiple assay plates:

Plate format	Plate count	cell#/ well	cell#/ mL	Cell suspension/ well [µL]	Total well# incl. extra wells	Required volume [mL]	Required cell count
96	1	$1x10^4$	$2.5x10^5$	40	120	4.8	$1.2x10^6$
	2				230	9.2	$2.3x10^6$
	3				320	12.8	$3.2x10^6$
	4				410	16.4	$4.1x10^6$
384	1	$2x10^3$	$2x10^5$	10	450	4.5	$9x10^5$
	2				800	8.0	$1.6x10^6$

Examples for cell counts per well with adapted volumes for the use of purified proteins, CCSN or plasma/serum:

Test item	purified protein / CCSN (20 µL)		plasma/serum (5 µL)	
Plate format	Cell# (1)	Cell#/mL (1)	Cell# (2)	Cell#/mL (2)
96	$1x10^4$ (in 40 µL)	$2.5x10^5$	$1x10^4$ (in 37.5 µL)	$2.67x10^5$
384	$2x10^3$ (in 10 µL)	$2x10^5$	$2x10^3$ (in 10 µL)	$2x10^5$

Volumes for 384-wells are not changed using mouse plasma/serum samples. The adjusted target cells are kept at RT until further use.

For plating, the effector and target cells are mixed and seeded within 30 min after target cell preparation.

Note: cell line PA-1-SC12 loses viability quickly outside the incubator, thus best processed quickly.

Assay Setup

4 Plating of effector and target cells

The cytotoxicity assay is performed in a 96- or 384-well luminescence plate. With unknown test

material, a transparent luminescence plate can be used or an additional clear cell-culture plate for microscopy analysis is prepared in the same manner to be able to observe cytotoxic effects (T-cell clustering, proliferation or target-cell lysis).
Usually, two time points are measured per assay (24 h and 48 h) i.e. each plate needs to be pipetted twice. If further relevant time points e.g. 72 h or 96 h more plates need to be prepared accordingly.

The adjusted cell suspensions with targets and effectors are first pooled in a volume ratio of 1:1 in an appropriate tube. The total volume results from the total well count. An adequate extra volume needs to be calculated (e.g. 10 mL suspension per 96-well plate/3 mL per 384-well plate). Target cells are adjusted to 2.5x105 cells/mL for 96-well plates, and 2x105 cells/mL for 384-well plates, respectively. The effector-target mixed cell suspension is plated as follows:
96-well format = 80 µL/well;
384-well format = 10 µL/well.
For analyzing mouse plasma/serum, the volume is reduced accordingly.
Example: 5 µL mouse plasma → 75 µL effector-target mixed cell suspension consisting of 37.5 µL effectors and 37.5 µL targets. The suspension is homogenized by inverting the tube multiple times and transferring the contents to a single-use, sterile reagent reservoir. Distribute cells promptly on the plates, preferably with an electric Eppendorf Xplorer®, manual multichannel pipette or Integra Voyager and the appropriate filter tips. The operating volume is set, and 100 µL or 25 µL DPBS or cell suspension is dispensed into empty wells in a 96-well or 384-well plate, respectively. An hour of incubation time before proceeding is recommended. For 384-well plates, an Integra Voyager pipette is used.
Note: 384-well plates are additionally sealed with a semipermeable membrane and centrifuged quickly at max. 300 xg by pressing the “short” button.

5 Dilution of test material

Test material (bsAb - purified, in CCSN or plasma/serum) is diluted in its storage buffer, Mock4 CCSN or assay medium, respectively. If available, the same volume of mock CCSN is added to the Lmin and Lmax control wells. Dilution rows are always prepared freshly by preparing a serial dilution starting with the highest and down to the lowest concentration.
Generally, for the first dilution at least 5 µL of the test item is used to reduce pipetting errors and falsified concentrations. If the protein concentration is known and high enough, a concentration as high as 5000 ng/mL may be used as the starting point at the investigator’s discretion for each experiment. Depending on the dilution volumes different 96-well plates or reaction cups are chosen to dilute the test items.
Up to 2 mL total volume per dilution step a plate can be used:

Protein solution per well	Dilution plate format
Up to 220 µL	96-well microtiter plate with round bottom
220 – 1000 µL	96-deep well plate 1000 µL
1000 – 2000 µL	96-deep well plate 2000 µL

Prior to use, all solutions need to be at RT to ensure that the assay requirements for temperature are complied. For optimal mixing of proteins in diluent, the sample is mixed multiple times with an appropriate pipette (e.g. 1 mL protein dilution is mixed with a 1000 µL pipette adjusted to 500 µL). It is also possible to use a suitable pipette i.e. Eppendorf Xplorer® with the function "pipette and mix". If only one concentration of test item is required, the dilution can be prepared in a suitable reaction tube. Prepared dilutions are stored at RT and used within 30 min; otherwise, storage is at 2 – 8 °C. Dilutions can be stored at RT for 2 h max., since proteins may bind to the surface and mask the actual concentration. If available, a standard reference is prepared in the protein concentration range (e.g. 10-point, 5-step dilution from 5000 – 0.00256 ng/mL). This serves as positive control and intra-assay comparison between different plates. If no reference is available e.g. for new target proteins, no intra-assay comparison can be done.

6 Addition of test material

The pre-diluted test items in multi-well plates can be added to the assay plate(s) using an electric Eppendorf Xplorer® Multichannel Pipette, manual Eppendorf multichannel or Integra Voyager. If the test item is prepared in a single tube, a manual or electric dispenser (e.g. Multipette Xstream® with a 1 mL Combitip advanced® plus) is used. 20 µL (96-well) or 5 µL (384-well) of test item and equal amounts of buffer or mock medium is added to each corresponding L_{min} and L_{max} wells. If only low amounts of test material are available with no extra volume to consider, 20 µL or 5 µL test item is then added with an appropriate single channel pipette. The tips have to be changed after the test item addition to each well/row/column. For the analysis of mouse plasma/serum, 75 µL/20 µL E:T cell mixture is added. During in vivo cytotox planning the sample concentration is determined by the investigator. Mouse serum/plasma can be used as percentage from 1 – 5 % per well or as defined concentration after Gyros ELISA quantification. If using defined mouse sample concentrations, the reference protein dilutions are used correspondingly. Subsequently diluted or undiluted mouse test item (1 – 5 µL, according experiment plan) is added. IF less than 5 µL serum/plasma are used, they are diluted with assay medium up to 5 µL. The pre-dilutions with can be prepared in 96-well round bottom plates and be directly transferred with an appropriate multi-channel pipette.
Tabular overview about the components' volumes per well:

Analysis for:	96-well		384-well	
	protein/ CCSN	mouse plasma-/serum	protein/ CCSN	mouse plasma-/serum
Effector + target [µL]	80	75	20	20
Test item or controls [µL]	20	1 – 5 µL topped up to 5 µL	5	1 – 5 µL topped up to 5 µL
Protein buffer or neg. serum/plasma [µL]	N/A	20	N/A	5
Final volume/well	**100 µL**	**100 µL**	**25 µL**	**30 µL**
Serum or plasma concentration	N/A	1 – 5 %	N/A	3.3 – 16.7 %

Moreover, negative control serum/plasma is added correspondingly to the reference protein dilutions, as well as the protein buffer is added to the mouse samples.
Example for 96-well:
Control: 3 µL reference protein
+ 5 µL Mock serum/plasma/CCSN
+ 17 µL assay medium
+ 75 µL cells
= 100 µL total volume
Samples: 5 µL serum/plasma/CCSN
+ 3 µL storage buffer
+ 17 µL assay medium
+ 75 µL cells
= 100 µL total volume

Note: 384-well plates are additionally sealed with a semipermeable membrane and centrifuged quickly at max. 300 xg by pressing the “short” button.

Incubation

7 Assay plates ready for analysis are placed in an incubator set at 37 °C with 5 % CO^2 and saturated humidity and removed only at the designated time points e.g. 24 h, 48 h etc. for reading on a luminometer.

Luminescence Measurement

8 All following steps are performed under non-sterile conditions. The Lmax-defined wells (maximal lysis) get 10 µL (96-well) or 5 µL (384-well) of 20 % Triton™ X-100 lysis solution with an electric Xplorer® pipette, or a suitable 8-channel manual pipette or Integra Voyager, adjusted to dispense

10 µL/5 µL. For maximal effects the wells are mixed by forward-reverse pipetting. The “pipette and mix” mode can be used on Xplorer®. The plate is placed in the incubator for further 10 min. During the Triton™X-100 incubation time, luciferin solution is prepared in a light-protected 15 mL tube (amber or aluminum foil-wrapped). The solution is transferred by decanting into a reagent reservoir and the residual solution added by pipetting. Quickly afterwards 20 µL luciferin solution per 96- well and 12.5 µL per 384-well are distributed onto the assay plates, respectively, preferably with an electric Xplorer® Multichannel pipette (mode “dispense”) or Integra Voyager. Start adding luciferin into column 1, rows A – H and proceed uniformly with an 8-channel manual pipette. All steps need to be proceeded with quickly. The finished plate is incubated immediately in an incubator for 30 min, protected from light. Handle a maximum of four 96-well plates or two 384-well plates at the same time.

The measurement must be performed 5 min after the 30-min incubation time ends e.g. with an Infinite M200 Pro or F200 Pro luminescence reader with Tecan i-control software.

Workflow

9 Workflow for luciferase-based cytotoxic assay protocol