Angiopoietin-2 ELISA

for the quantitative determination of human Angiopoietin-2 in serum, EDTA plasma, heparin plasma, and citrate plasma

Cat. No. BI-ANG2 . 12 x 8 tests

FOR RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES

CONTENTS

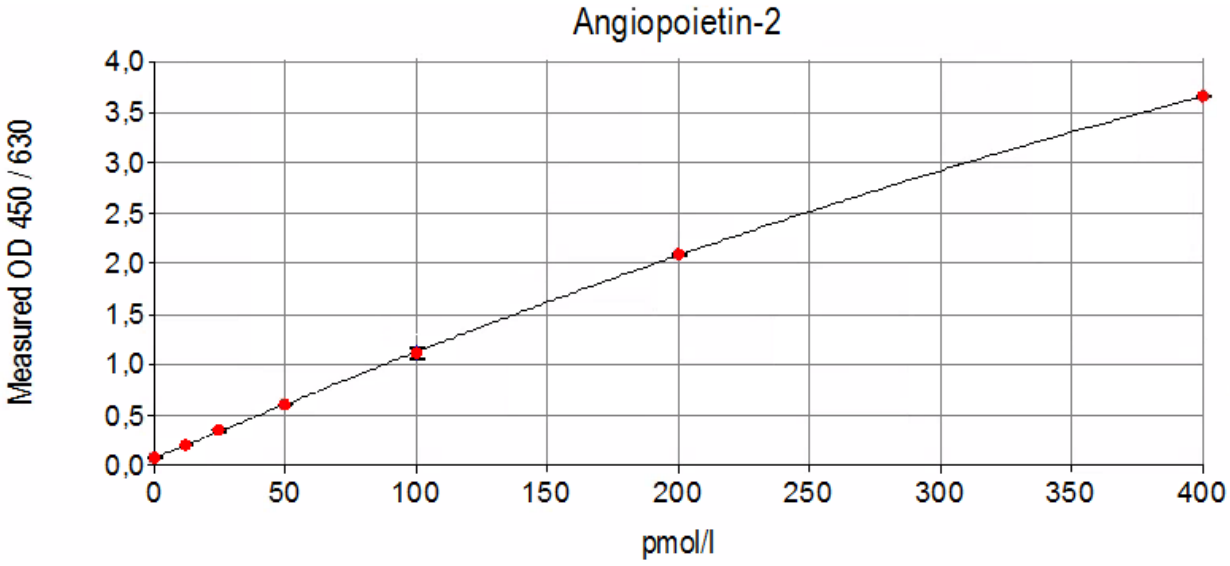
ASSAY CHARACTERISTICS Summary

Method	Sandwich ELISA, HRP/TMB, 12x8-well detachable strips			
Sample type(s)	Serum, EDTA plasma, citrate plasma, heparin plasma			
Sample volume	20 µl / sample			
Assay time	2 h / 1 h / 30 min			
Sensitivity	LOD: 3.7 pmol/l (=203 pg/ml); LLOQ: 6.3 pmol/l (=346 pg/ml)			
Standard range	0 – 400 pmol/l (0 / 12.5 / 25 / 50 / 100 / 200 / 400)			
Conversion factor	1 pg/ml = 0.018 pmol/l; MW: 54.9 kDa			
Precision		**n**	**CV (%)**	
	Within-run	3	≤8	
	In-between-run	9	≤6	
Accuracy (Spike/Recovery of recombinant ANG2)		**n**	**Recovery (%)**	
			+36 pmol/l	**+180 pmol/l**
	Serum	6	81	93
	Citrate plasma	1	100	95
			+40 pmol/l	**+200 pmol/l**
	EDTA plasma	6	85	78
	Heparin plasma	1	89	79
Dilution linearity of endogenous Angiopoietin-2		**n**	**Recovery of expected dilution (%)**	
			1+1	**1+3**
	Serum	6	105	105
	EDTA plasma	6	110	122
	Heparin plasma	1	107	109
	Citrate plasma	1	104	115
Specificity*	Endogenous and recombinant human Angiopoietin-2.			
Use	Research use only.			
Values of apparently healthy donors		**n**	**Median Angiopoietin-2 (pmol/l)**	
	Serum	11	28	
	EDTA plasma	11	24	
	Heparin plasma	11	25	
	Citrate plasma	11	23	

*: according to epitope mapping and sequence analysis the Angiopoietin-2 ELISA should detect all 3 Angiopoietin-2 isoforms. No cross-reactivity with Angiopoietin-1.

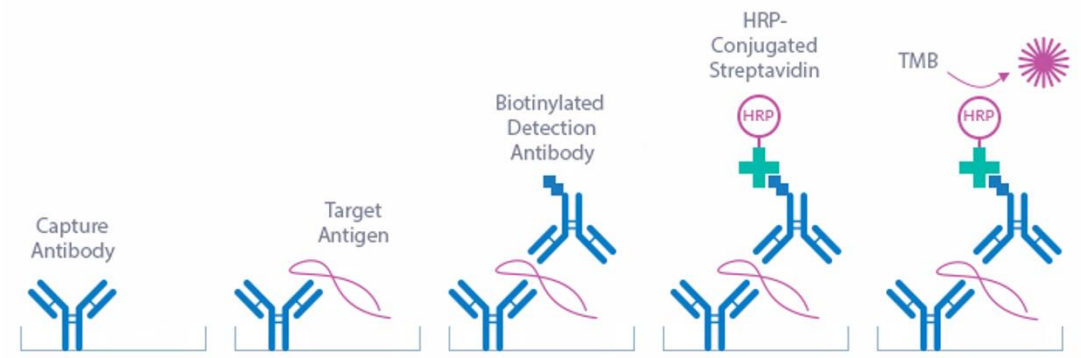
For further information on assay performance characteristics, matrix comparisons and stability data please find data in this validation data file or contact our customer service by e-mail info@bmgrp.com or by phone +43/1/29107-45.

TYPICAL STANDARD CURVE



PRINCIPLE OF THE ASSAY

The Angiopoietin-2 human ELISA kit is a sandwich enzyme immunoassay for the quantitative determination of Angiopoietin-2 in human serum and plasma samples. Standards, controls and samples must be pre-diluted 1+10 prior to assaying. In a first step, pre-diluted standard/control/sample and biotinylated antibody (goat polyclonal anti-human Angiopoietin-2) are pipetted into the wells of the microtiter strips, which are pre-coated with a monoclonal anti-human Angiopoietin-2 antibody. Angiopoietin-2 present in the standard/control/sample binds to the pre-coated antibody in the well and forms a sandwich with the anti-human Angiopoietin-2 antibody. In a washing step all non-specific unbound material is removed. In a second step, the conjugate (Streptavidin-HRP) is pipetted into the wells and reacts with the biotinylated antibody. After another washing step, the substrate (TMB, tetramethylbenzidine) is pipetted into the wells. The enzyme-catalyzed color change of the substrate is directly proportional to the amount of Angiopoietin-2 present in the sample. This color change is detectable with a standard microplate reader. A dose response curve of the absorbance (optical density, OD at 450 nm) using the values obtained from the standards versus the standard concentration is generated. The concentration of Angiopoietin-2 in the sample is determined directly from the dose response curve.

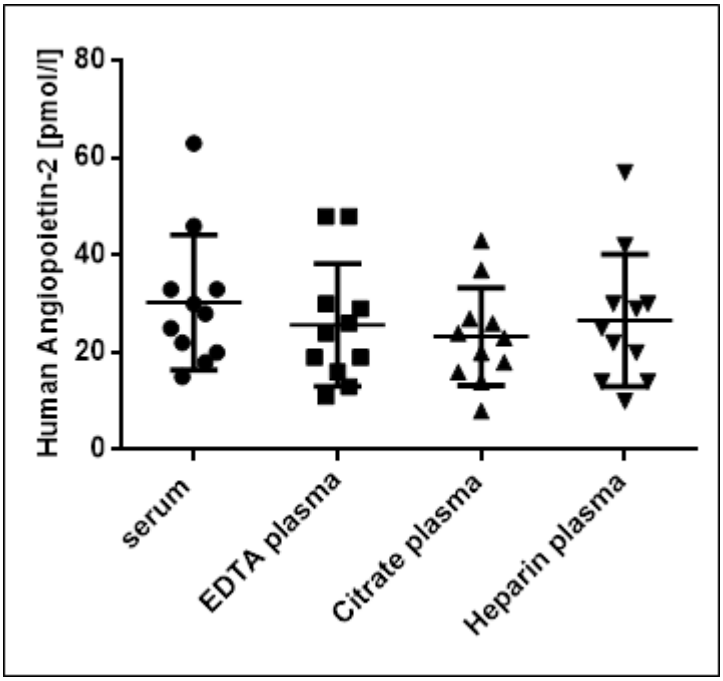


SAMPLE VALUES

Angiopoietin-2 Values in Apparently Healthy Individuals

To provide values for circulating Angiopoietin-2 (ANG2), a panel of samples from apparently healthy donors was tested. Each individual donated blood for all tested sample matrices.

	ANG2 [pmol/l]			
	Serum	**EDTA plasma**	**Citrate plasma**	**Heparin plasma**
# of samples	n=11	n=11	n=11	n=11
Mean	30.27	25.73	23.27	26.64
Median	**28**	**24**	**23**	**25**
5% Percentile	15	11	8	10
95% Percentile	63	48	43	57
Minimum	15	11	8	10
Maximum	63	48	43	57



It is recommended to establish the normal range for each laboratory.

Angiopoietin-2 Values in Disease Panels

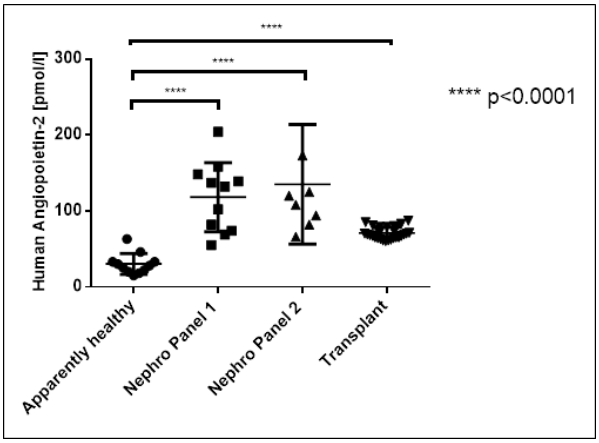
In addition to samples from apparently healthy donors, panels of samples from chronic kidney disease (CKD) patients, CKD patients on dialysis, CKD patients with a kidney transplant (NTX), as well as an unselected hospital panel were tested.

Summary of the results obtained with several disease panels:

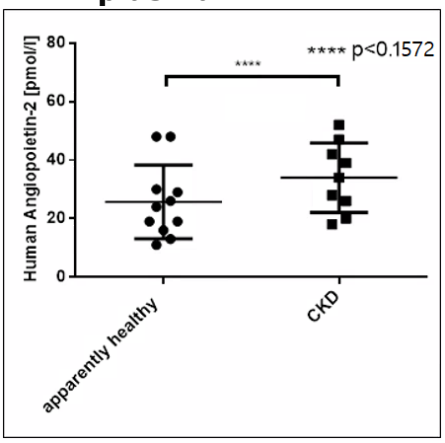
	Angiopoietin-2 [pmol/l]			
	Serum app. healthy	**Serum, Nephro#1**	**Serum Nephro#2**	**Serum, NTX**
# of samples	11	n=11	n=8	n=29
Mean	30.27	118.2	135.1	70.86
Median	**28**	**132**	**114**	**69**
Minimum	15	55	66	60
Maximum	63	204	313	87

Comparison of ANG2 Values in Apparently Healthy Individuals and Disease Panels

Serum



EDTA plasma

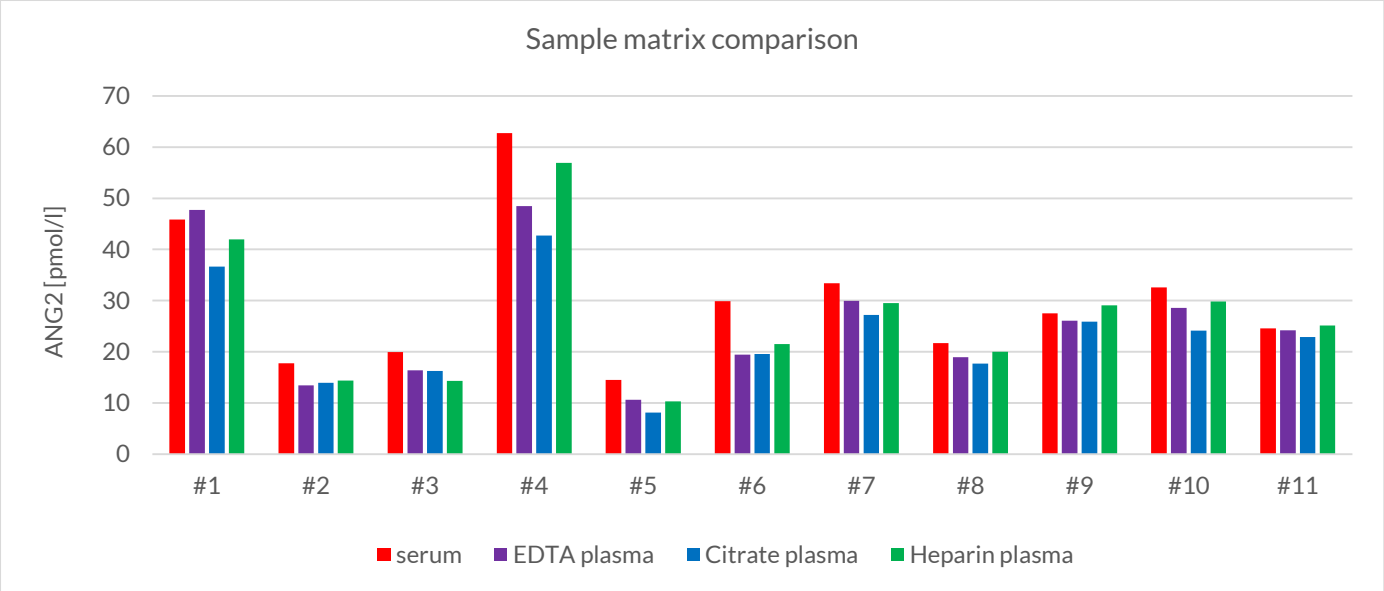


MATRIX COMPARISON

To assess whether all tested matrices behave the same way in the Angiopoietin-2 (ANG2) ELISA, concentrations of ANG2 were measured in serum, EDTA, heparin, and citrate plasma samples prepared from 11 apparently healthy donors. Each individual donated blood in all tested sample matrices.

Data and graph for apparently healthy donors:

Donor ID	**Angiopoietin-2 [pmol/l]**			
	Serum	**EDTA plasma**	**Citrate plasma**	**Heparin plasma**
#1	46	48	37	42
#2	18	13	14	14
#3	20	16	16	14
#4	63	48	43	57
#5	15	11	8	10
#6	30	19	20	22
#7	33	30	27	30
#8	22	19	18	20
#9	28	26	26	29
#10	33	29	24	30
#11	25	24	23	25



ASSAY PERFORMANCE CHARACTERISTICS

ACCURACY

The accuracy of an ELISA is defined as the precision with which it can recover samples of known concentrations.

The recovery of the Angiopoietin-2 (ANG2) human ELISA was measured by adding recombinant ANG2 to samples containing a known concentration of endogenous ANG2. The %recovery of the spiked concentration was calculated as the percentage of measured compared over the expected value.

This table shows the summary of the recovery experiments in the Angiopoietin-2 human ELISA in different sample matrices:

Sample matrix	n	Spike/Recovery [%]			
		+36 pmol/l		+180 pmol/l	
		Mean	Range	Mean	Range
Serum	6	81	72-94	93	77-100
Citrate plasma	1	100	-	95	-

Sample matrix	n	Spike/Recovery [%]			
		+40 pmol/l		+200 pmol/l	
		Mean	Range	Mean	Range
EDTA plasma	6	85	73-94	78	71-85
Heparin plasma	1	89	-	79	-

Experiments:
Recovery of spiked samples was tested by adding 2 concentrations of human recombinant ANG2 to different human sample matrices.

Data showing recovery of recombinant ANG2 in human serum samples:

Sample ID	Spike ANG2 [pmol/l]			S/R [%]	
	0	36	180	36	180
S1	18	51	181	88	96
S2	20	52	185	94	97
S3	63	84	202	76	95
S4	15	39	147	72	77
S5	30	55	195	78	100
S6	33	58	187	78	95
			Mean S/R [%]	**81**	**93**
			Min	72	77
			Max	94	100

Data showing recovery of recombinant ANG2 in human citrate plasma samples:

Sample ID	Spike ANG2 [pmol/l]			S/R [%]	
	0	36	180	36	180
C1	16	51	180	**100**	**95**

Data showing recovery of recombinant ANG2 in human EDTA plasma samples:

Sample ID	Spike ANG2 [pmol/l]			S/R [%]	
	0	40	200	40	200
E1	13	45	164	81	79
E2	16	50	168	87	80
E3	48	78	166	87	71
E4	11	47	160	94	77
E5	19	53	180	89	85
E6	30	56	164	73	74
			Mean S/R [%]	**85**	**78**
			Min	73	71
			Max	94	85

Data showing recovery of recombinant ANG2 in human heparin plasma samples:

Sample ID	Spike ANG2 [pmol/l]			S/R [%]	
	0	40	200	40	200
H1	14	49	165	**89**	**79**

DILUTION LINEARITY & PARALLELISM

Tests of dilution linearity and parallelism ensure that both recombinant and endogenous samples containing Angiopoietin-2 (ANG2) behave in a dose dependent manner and are not affected by matrix effects. Dilution linearity assesses the accuracy of measurements in diluted human samples spiked with known concentrations of recombinant analyte. By contrast, parallelism refers to dilution linearity in human samples and provides evidence that the endogenous analyte behaves in the same way as the recombinant one. Dilution linearity and parallelism are assessed for each sample type and should be within 20% of the expected concentration.

Parallelism

Experiment:
Parallelism was assessed by serially diluting human samples containing **endogenous** ANG2 with assay buffer.

Summary table below shows the mean recovery and range of serially diluted endogenous ANG2 in several sample matrices:

Sample matrix	n	Recovery [%]			
		1+1		1+3	
		Mean	Range	Mean	Range
Serum	6	105	98-115	105	98-120
EDTA plasma	6	110	100-128	122	110-139
Citrate plasma	1	104	-	115	-
Heparin plasma	1	107	-	109	-

Data showing dilution linearity of endogenous ANG2 in human serum samples:

Sample ID	ANG2 [pmol/l]			Recovery [%]	
	Ref	1+1	1+3	1+1	1+3
S1	73	42	22	115	120
S2	81	42	21	104	101
S3	87	42	21	98	99
S4	80	43	22	106	109
S5	83	43	20	104	98
S6	79	41	20	104	101
			Mean R [%]	**105**	**105**
			Min	98	98
			Max	115	120

Data showing dilution linearity of endogenous ANG2 in human EDTA plasma samples:

Sample ID	ANG2 [pmol/l]			Recovery [%]	
	Ref	1+1	1+3	1+1	1+3
E1	76	48	25	128	133
E2	81	45	24	111	120
E3	72	36	20	101	110
E4	67	35	20	105	118
E5	67	34	19	100	115
E6	52	29	18	112	139
			Mean R [%]	**110**	**122**
			Min	100	110
			Max	128	139

Data showing recovery of endogenous ANG2 in a human citrate plasma sample:

Sample ID	ANG2 [pmol/l]			Recovery [%]	
	Ref	1+1	1+3	1+1	1+3
C1	117	61	33	104	115

Data showing recovery of endogenous ANG2 in a human heparin plasma sample:

Sample ID	ANG2 [pmol/l]			Recovery [%]	
	Ref	1+1	1+3	1+1	1+3
H1	160	85	44	107	109

Dilution Linearity

Experiment:
Dilution linearity was assessed by serially diluting samples containing **recombinant** ANG2 with assay buffer.

Summary table below shows the mean recovery and range of serially diluted recombinant ANG2 in several sample matrices:

Sample matrix	n	Recovery [%]			
		1+1		1+3	
		Mean	Range	Mean	Range
Serum	6	120	112-141	124	117-140
EDTA plasma	6	106	103-110	122	114-131
Citrate plasma	1	118	-	117	-
Heparin plasma	1	97	-	111	-

Data showing dilution linearity of recombinant ANG2 in human serum samples:

Sample ID	ANG2 [pmol/l]			Recovery [%]	
	Ref	1+1	1+3	1+1	1+3
S1	181	104	54	116	119
S2	185	108	56	117	122
S3	202	114	60	112	118
S4	147	103	51	141	140
S5	195	112	57	115	117
S6	187	114	60	122	127
			Mean R [%]	**120**	**124**
			Min	112	117
			Max	141	140

Data showing recovery of recombinant ANG2 in a human EDTA plasma sample:

Sample ID	ANG2 [pmol/l]			Recovery [%]	
	Ref	1+1	1+3	1+1	1+3
E1	164	86	49	105	119
E2	168	93	51	110	121
E3	166	86	54	103	130
E4	160	87	46	109	116
E5	180	93	51	104	114
E6	164	87	53	106	131
			Mean R [%]	**106**	**122**
			Min	103	114
			Max	110	131

Data showing recovery of recombinant ANG2 in a human citrate plasma sample:

Sample ID	ANG2 [pmol/l]			Recovery [%]	
	Ref	1+1	1+3	1+1	1+3
C1	173	102	51	118	117

Data showing dilution linearity of recombinant ANG2 in human serum samples:

Sample ID	ANG2 [pmol/l]			Recovery [%]	
	Ref	1+1	1+3	1+1	1+3
H1	165	80	46	97	111

PRECISION

The precision of an ELISA is defined as its ability to measure the same concentration consistently within the same experiments carried out by one operator (within-run precision or repeatability) and across several experiments using the same samples but conducted by several operators at different locations using different ELISA lots (in-between-run precision or reproducibility).

Within-Run Precision (Intra-Assay)

Experiment:
2 samples of known concentrations were tested 3 times within 1 kit lot by 1 operator.

Within-run (n=3)	Sample 1	Sample 2
Mean (pmol/l)	25	201
SD (pmol/l)	2.1	3.0
CV (%)	8	1

In-Between-Run Precision (Inter-Assay)

Experiment:
2 samples of known concentrations were tested 9 times within 2 kit lots by 2 operators.

In-between-run (n=9)	Sample 1	Sample 2
Mean (pmol/l)	26	201
SD (pmol/l)	1.6	5.1
CV (%)	6	3

DETECTION LIMIT & SENSITIVITY

To determine the sensitivity of the Angiopoietin-2 (ANG2) ELISA, experiments measuring the lower limit of detection (LOD) and the lower limit of quantification (LLOQ) were conducted.

The LOD, also called the detection limit, is the lowest point at which a signal can be distinguished above the background signal, i.e. the signal that is measured in the absence of ANG2, with a confidence level of 99%. It is defined as the mean back calculated concentration of standard 1 (0 pmol/l of ANG2, five independent measurements) plus three times the standard deviation of the measurements.

The LLOQ, or sensitivity of an assay, is the lowest concentration at which an analyte can be accurately quantified. The criteria for accurate quantification at the LLOQ are an analyte recovery between 75% and 125% and a coefficient of variation (CV) of less than 25%. To determine the LLOQ, standard 2, i.e. the lowest standards containing recombinant ANG2, is diluted, measured five times and its concentration back calculated. The lowest dilution, which meets both criteria, is reported as the LLOQ.

The following values were determined for the Angiopoietin-2 ELISA:

LOD	**3.7 pmol/l**
LLOQ	**6.3 pmol/l**

SAMPLE STABILITY

Sample Collection and Storage

Serum, EDTA plasma, heparın plasma, and citrate plasma are suitable for use in this assay. Do not change sample type during studies. We recommend duplicate measurements for all samples, standards and controls. The sample collection and storage conditions listed are intended as general guidelines.

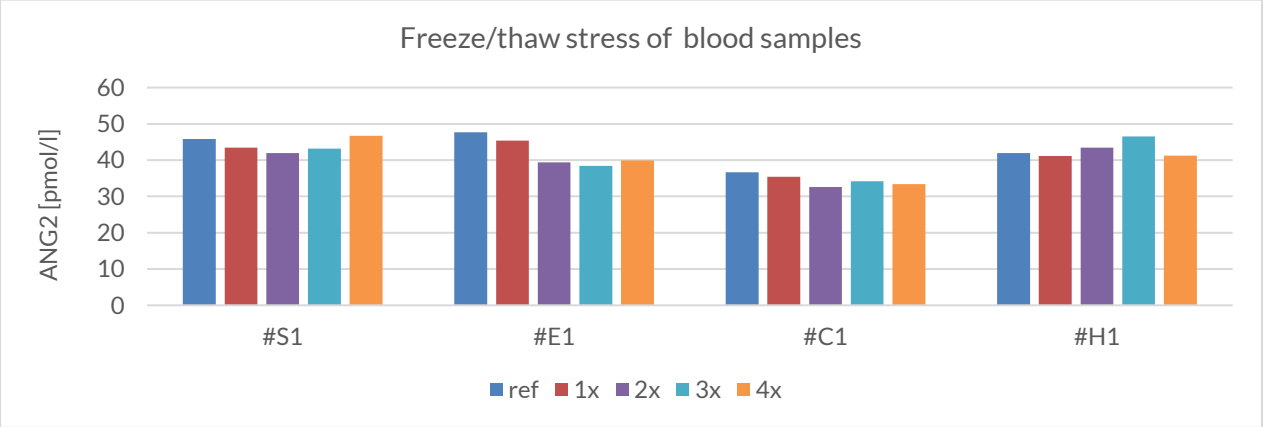
Serum & Plasma
Collect venous blood samples in standardized blood collection tubes. Perform plasma or serum separation by centrifugation as soon as possible according to the tube manufacturer's instructions for use. Assay acquired samples immediately or aliquot and store at -25°C or lower. Lipemic or haemolyzed samples may give erroneous results. Samples are stable for at least four freeze-thaw cycles.

Freeze-Thaw Stability of Samples Containing Endogenous Human Angiopoietin-2

The stability of endogenous Angiopoietin-2 (ANG2) was tested by comparing four measurements in samples that had undergone four freeze-thaw cycles (F/T).

For freeze-thaw experiments, samples were collected according to the supplier's instruction using blood collection devices and stored at -80°C. Reference samples were freeze-thawed once. The mean recovery of sample concentration after four freeze-thaw cycles is 94%.

	ANG2 [pmol/l]					Recovery [%] 4 F/T cycles
Sample ID	**Ref**	**1x**	**2x**	**3x**	**4x**	
Serum #1	46	43	42	43	47	102
EDTA plasma #1	48	45	39	38	40	84
Citrate plasma #1	37	35	33	34	33	91
Heparin plasma #1	42	41	43	47	41	98
					Mean R [%]	**94**



All samples should undergo a maximum of four freeze-thaw cycles.

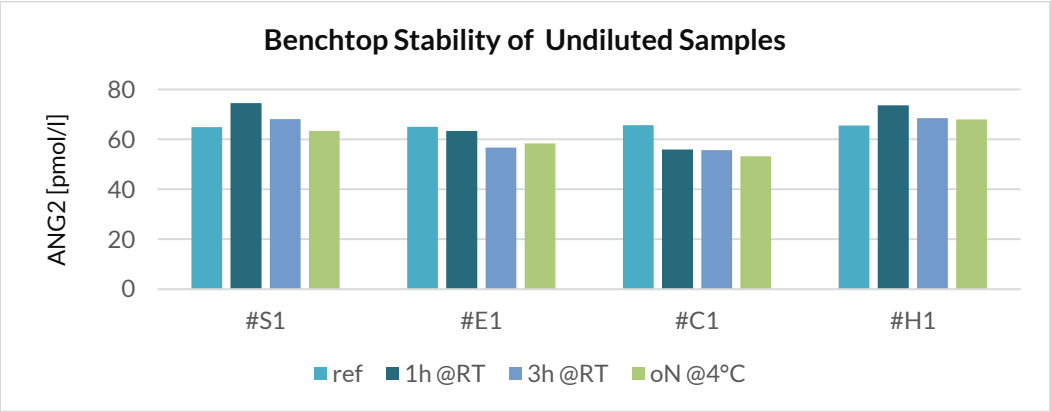
Benchtop Stability of Samples Containing Endogenous Angiopoietin-2

The benchtop stability of endogenous Angiopoietin-2 was tested by comparing Angiopoietin-2 measurements in human samples that had been stored at different temperatures.

For the assessment of the benchtop stability, a set of undiluted human samples was aliquoted and stored at at room temperature or at 4°C. Samples can be stored for at least three hours at room temperature as well as overnight at 4°C. The mean recovery of sample concentrations after overnight storage at 4°C is 93%.

Angiopoietin-2 concentrations of samples stored at -25°C (reference), at room temperature (RT) or overnight (oN) at 4°C:

	Angioppoietin-2 [pmol/l]				R [%]
Sample ID	**Ref**	**1 h RT**	**3 h RT**	**oN 4°C**	**oN 4°C vs ref**
#S1	65	75	68	63	98
#E1	65	63	57	58	90
#C1	66	56	56	53	81
#H1	66	74	68	68	104
				Mean R [%]	**93**



SPECIFICITY

This assay recognizes endogenous and recombinant human Angiopoietin-2. According to epiptope mapping and sequence analysis Angiopoietin-2 ELISA should detect all 3 isoforms. Angiopoietin-1 is not detected in this assay.

The specificity of an ELISA is defined as its ability to exclusively recognize the analyte of interest. The specificity of the human Angiopoietin-2 ELISA was shown by characterizing both the capture and the detection antibody through epitope mapping (Figure 1-3).
Both antibodies used in the human Angiopoietin-2 ELISA bind to Angiopoietin-2 with high affinity.
Moreover, the specificity of the ELISA was established through competition experiments, which measure the ability of the antibodies to exclusively bind to Angiopoietin-2.

Characterization of the Antibodies

CAB Coating Antibody: recombinant monoclonal mouse anti-human Angiopoietin-2
DAB Detection Antibody: polyclonal goat anti-human Angiopoietin-2

Epitope Mapping

Antibody binding sites were determined by epitope mapping using microarray analysis (Pepperprint GmbH). The capture antibody shows no linear epitope, but has a structural epitope which binds to receptor binding site of human ANG2. This receptor binds to TEL/TIE2 protein.
The detection antibody binds to several epitopes distributed over the entire Angiopoietin-2 molecule.

Epitope mapping of detection antibody

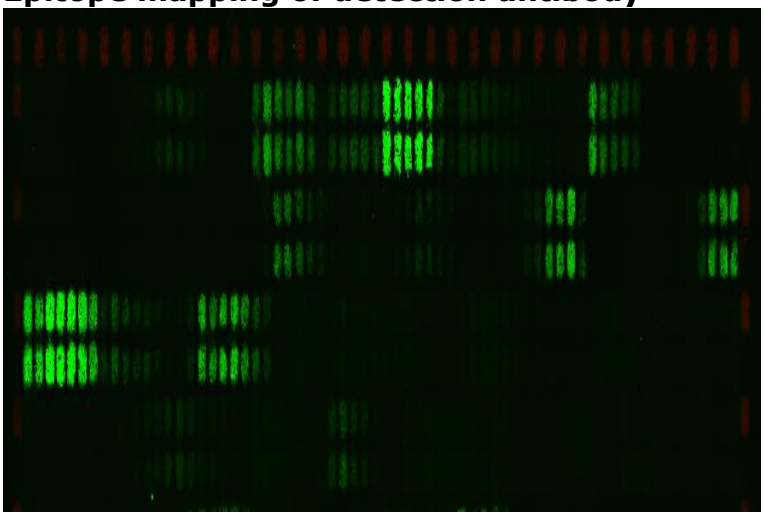


Fig. 1: Microarray of polyclonal detection antibody reveals seven linear epitopes on human ANG-2 sequence. Fluorescent signals (green) illustrate where the antibody binds to peptide sequences on a microarray chip. These peptides were spotted as 15mers with an 13 amino acid overlap in duplicates on the chip and represent the whole human Angiopoietin-2 sequence. Red fluorescent signals mark the position of control peptides

High affinity antibodies

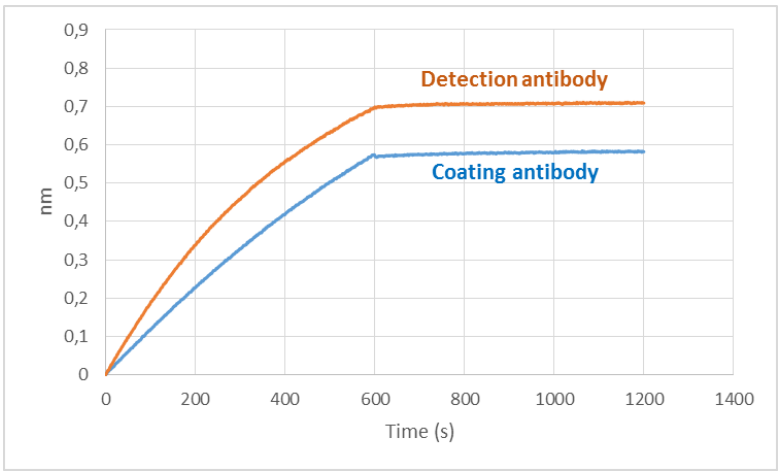


Fig. 2: Biolayer interferometry binding kinetics demonstrate low dissociation of both coating (blue) and detection antibody (orange) towards human Angiopoietin-2 protein (k_{dis}<1.0E-07 s^{-1}) Association and dissociation were determined for 600s (x-axis).

Antibody epitopes on human ANG-2 molecule

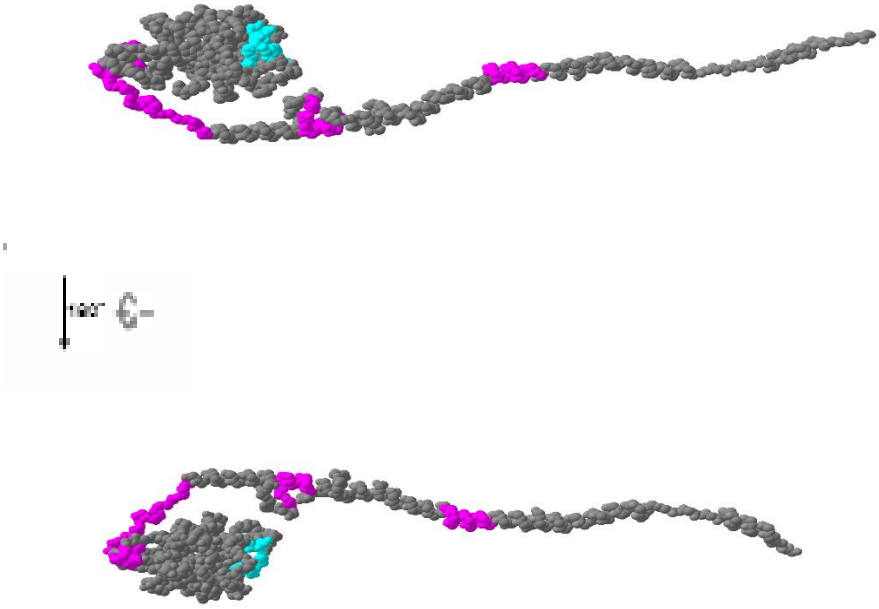


Fig. 3: SWISS model of human Angiopoietin-2 monomer (122-493 aa, O15113/ 3ghg.1.B) with antibody epitopes. The monoclonal coating antibody binds to the bioactive receptor-binding site within the C-terminus (structural epitope, blue). Linear epitopes (purple) of the detection antibody range over the whole protein. The N-terminus and three additional epitopes are not shown here.

Competition of Signal

Competition experiments were carried out by pre-incubating human samples containing endogenous levels of Angiopoietin-2 with an excess of capture antibody (CAB).
The concentration measured in this mixture was then compared to a reference value, which was obtained from the same sample but without the pre-incubation step.
Mean competition was 87%, and is within the range of acceptance of international guidelines.

ID	Angiopoietin-2 [pmol/l]		Recovery [%] Competition
	Reference	Reference + CAB	
Sample 1	295	17	94
Sample 2	36	7	81
Sample 3	66	14	79
Sample 4	103	16	84
Sample 5	141	24	83
Sample 6	398	3	99
Sample 7	157	19	88
		Mean Comp. [%]	**87**

CALIBRATION

This immunoassay is calibrated against recombinant human soluble Angiopoietin-2 protein (Uniprot ID O15123, https://www.uniprot.org/uniprot/O15123).

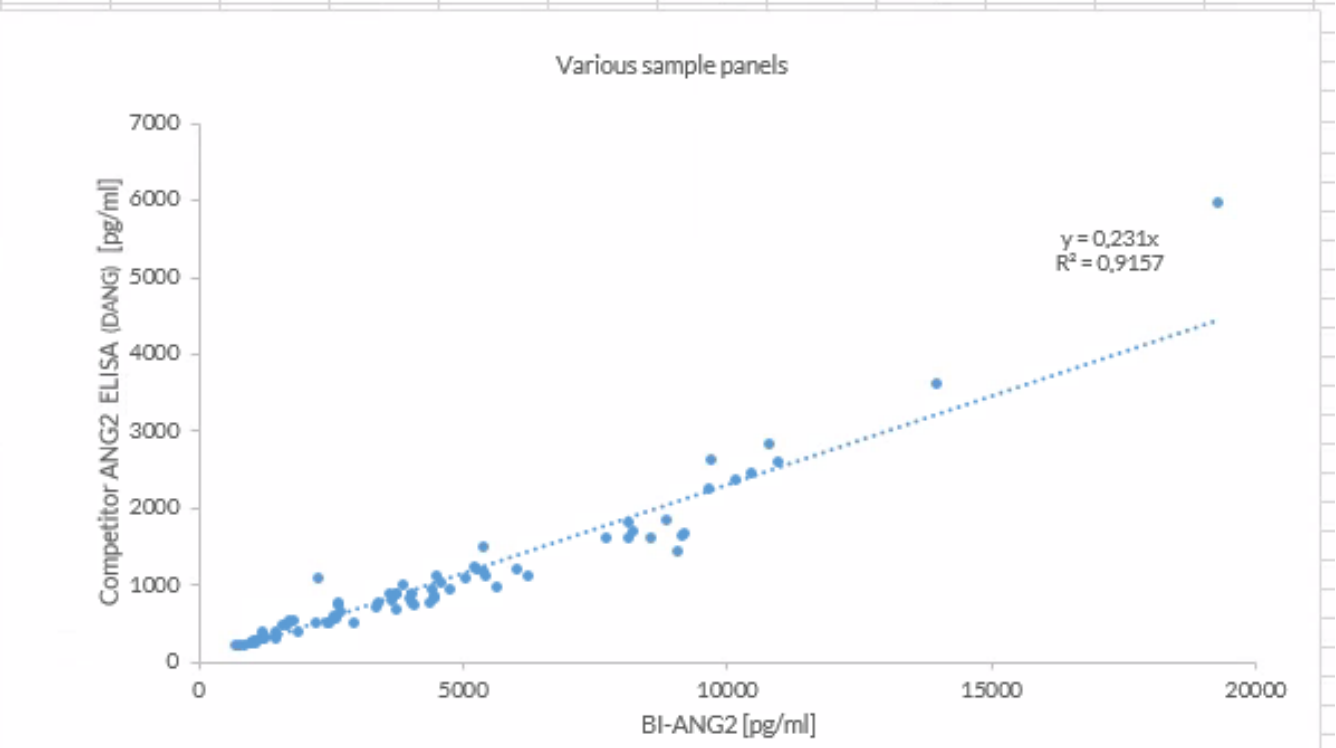
COMPARISON with other human ANGIOPOIETIN-2 ELISA assays

Assay Characteristics of different human Angiopoietin-2 ELISA assays

	BIOMEDICA	Another MANUFACTURER
Method	Sandwich ELISA,	Sandwich ELISA
Sample type	Plasma, serum, cell culture supernatant, urine	Plasma, serum, cell culture supernatant, urine, saliva
Sample volume	20 µl	20 µl
Assay time	3,5 h	4,5 h
Assay range	686 -*21,960* pg/ml (assay range optimized for clinical samples- no additional testing required)	46.9 - *3,000* pg/ml
Specificity	Endogenous and recombinant human ANG2. The ANG2 ELISA should detect all 3 ANG-2 isoforms	Natural and recombinant human ANG2
Antibodies	*Recombinant* human monoclonal ANG-2, polyclonal anti-human ANG2 (epitope-mapped antibodies)	monoclonal anti-human ANG2, monoclonal anti-human ANG2
Standard Matrix	*Human serum matrix* containting recombinant human ANG2 *7 ready to use standards*	Protein buffered matrix containing recombinant human ANG2 *1 stock standard vial*
Values of apparently healthy samples	Plasma mean value (n=11): 1317 pg/ml (604-2635 pg/l)	Plasma mean value (n=35): 1964 pg/ml (1071-4389 pg/ml)
Values of clinical samples	CKD:Serum mean value/range (n=32): 7105 pg/ml (2641-19,311 pg/ml)	not indicated
Controls	2 vials (high, low), included	not included
Validation	According to FDA/ICH/EMEA guidelines	not indicated
Use	RUO	RUO

Correlation of human samples measured with two different Angiopoietin-2 ELISA assay kits

The Biomedica human Angiopoietin-2 ELISA was compared with another human Angiopoietin-ELISA assay using serum and plasma samples from various cohorts (n=77). The correlation between the Biomedica and the competitor assay is R2 = 0.9157.



Sample panel:

- Cardio panel (n=16) – plasma
- Nephro panel (n= 32) - serum
- Control panel (apparently healthy) (n=77) - serum, plasma

Comparison of sample values [pg/ml] from a CKD-cohort measured with the Biomedica Angiopoietin-2 ELISA and an ELISA from another manufacturer

	BIOMEDICA human ANG2 ELISA		COMPETITOR human ANG2 ELISA
Sample ID	ANG2 [pmol/l]	ANG2 [pg/ml]	ANG2 [pg/ml]
S#1	168,3	9239	1644
S#2	200,4	11000	2565
S#3	98,8	5424	1482
S#4	98,5	5407	1169
S#5	255,2	14012	3603
S#6	167,1	9174	1630
S#7	81,9	4498	812
S#8	87,4	4796	916
S#9	68,8	3777	863
S#10	149,0	8180	1603
S#11	74,5	4091	873
S#12	75,2	4128	712
S#13	185,5	10182	2361
S#14	150,3	8253	1672
S#15	69,1	3791	673
S#16	67,6	3713	767
S#17	114,0	6256	1099
S#18	96,8	5316	1188
S#19	54,4	2988	490
S#20	80,8	4435	926
S#21	141,3	7757	1601
S#22	82,2	4514	826
S#23	148,9	8173	1795
S#24	191,5	10513	2430
S#25	156,6	8598	1582
S#26	162,0	8894	1824
S#27	48,1	2641	532
S#28	93,0	5106	1064
S#29	176,3	9681	2226
S#30	165,7	9098	1409
S#31	351,8	19311	5953
S#32	80,3	4408	737
	mean	**7105**	**1532**
	n	**16**	

Nephrologie CKD panel (n=32) -serum :
Biomedica mean values: 7105 +/- 635 pg/ml
Competotor mean values: 1532 +/-185 pg/ml

REFERENCES & DOCUMENTS

Validation Literature

The assay is fully validated according to:

1. ICH Topic Q2 (R1) „Validation of Analytical Procedures: Text and Methodology”
2. EMEA/CHMP/EWP/192217/2009 Guideline on bioanalytical method validation
3. Bioanalytical Method Validation, Guidance for Industry, FDA, May 2018

Additional Documents Available Online (www.bmgrp.com)

Instructions for Use (IFU, package insert)
Material Safety Data Sheet (MSDS)