Version 07-11/24

User's Manual

Human Adiponectin ELISA Kit

REF **DEIA-NB24-02**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

The Human Adiponectin ELISA Kit is intended to be used for quantitative measurement of human Adiponectin in human serum and plasma samples.

General Description

Adiponectin is a 30kDa protein which percentage in serum proteins is 0.01%. It is mainly synthesized by adipocytes, but also muscle cells and hepatocytes have the ability to synthesize Adiponectin. It consists of a Collagen-like N-terminal and a globular C-terminal domain. In vivo Adiponectin appears with different oligomers. Beside the trimer and ditrimer also high molecular multimers exist. Two different receptors are known, both receptors are ubiquitary expressed, though the distribution in the tissues varies. The Adiponectin Receptor 1 (AdipoR1) is especially in muscle- and AdipoR2 in liver tissue synthesized.

Several studies show, that adiponectin correlates negatively with BMI and thus it could have relevance for the energy metabolism for example through the regulation of fatty acid oxidation. Beside the correlation with BMI, Adiponectin level is associated with the Insulin-Resistance and so also linked with Type II Diabetes. Adiponectin is associated also with glucose- und lipometabolism.

Furthermore adiponectin is involved in inflammatory processes and therewith it is of importance for appearance of arteriosclerosis and coronaritis, thus the determination of Adiponectin level in plasma could serve to estimate the risk of coronary disease. Beside this Adiponectin influences further physiological processes as for example the angiogenesis.

Principles of Testing

The Human Adiponectin ELISA Kit is a so-called Sandwich-Assay using two specific and highly affine antibodies. The Adiponectin in the samples binds to the first antibody coated on the microtiter plate. In the following step the second specific anti-Adiponectin-Antibody binds in turn to the immobilised Adiponectin. The second antibody is biotinylated and will be applied in a mixture with a Streptavidin-Peroxidase-Enzyme Conjugate. In the closing substrate reaction, the turn of the colour will be catalysed quantitatively depending on the Adiponectin level of the samples.

Reagents And Materials Provided

1. **Microtiter plate:** ready for use, coated with mouse-anti-Adiponectin-antibody. Wells are separately breakable. (8x12) wells
2. **Calibrators A-E:** lyophilized, (native human Adiponectin), concentrations are given on vial labels and on quality certificate in ng/mL. 5 x 750 µL
3. **Control 1 (CTR1):** lyophilized, (human serum), concentration is given on quality certificate in ng/mL. 1 x 500 µL
4. **Control 2 (CTR2):** lyophilized, (human serum), concentration is given on quality certificate in ng/mL. 1 x 500 µL
5. **Antibody-HRP-Conjugate (DET):** ready for use, mouse-anti-hAdiponectin-antibody biotinylated +

streptavidin horseradish peroxidase conjugate. 1 x 12 mL

6. **Dilution Buffer (DIL):** ready for use. 1 x 125 mL
7. **Washing Buffer (WB):** 20-fold concentrated solution. 1 x 50 mL
8. **Substrate (S):** ready for use, horseradish-peroxidase-(HRP) substrate, stabilised Tetramethylbencidine. 1 x 12 mL
9. **Stopping Solution (STP):** ready for use, 0.2 M sulfuric acid. 1 x 12 mL
10. **Sealing Tape:** for covering the microtiter plate. 2

Materials Required But Not Supplied

1. Distilled (Aqua destillata) or deionized water for dilution of the Washing Buffer WB (A. dest.), 950 mL.
2. Precision pipettes and multichannel pipettes with disposable plastic tips
3. Polyethylene PE/Polypropylene PP tubes for dilution of samples
4. Vortex-mixer
5. Microtiter plate shaker (350 rpm)
6. Microtiter plate washer (recommended)
7. Microplate reader ("ELISA-Reader") with filter for 450 and ≥590 nm

Storage

Store the kit at 2-8°C after receipt until its expiry date. The lyophilized reagents should be stored at –20 °C after reconstitution. Avoid repeated thawing and freezing.

The shelf life of the components after initial opening is warranted for 4 weeks, store the unused strips and microtiter wells airtight together with the desiccant at 2-8°C in the clip-lock bag, use in the frame provided. The reconstituted components calibrators A-E and Controls CTR1 and CTR2 must be stored at –20°C (max. 4 weeks). For further use, thaw quickly but gently (avoid temperature increase above room temperature and avoid excessive vortexing). Up to 3 of the freeze-thaw cycles did not influence the assay. The 1:20 diluted Washing Buffer WB is 4 weeks stable at 2-8°C

Specimen Collection And Preparation

1. Sample type: Serum and Plasma

Serum and heparin plasma levels are comparable. In EDTA- and Citrate Plasma-samples levels were found approx. 18% lower, because of the relatively high amount of anticoagulant.

2. Specimen collection

The blood sample for serum preparation should be gained according to standardized venipuncture procedure. Hemolytic reactions have to be avoided.

3. Required sample volume: 10 µL

4. Sample stability

In firmly closable sample vials

• Storage at 20-25°C: 2 days

• Storage at -20° C: min. 2 years

• Freeze-thaw cycles max. 3

The storage of samples over a period of 2 years at -20°C, showed no influence on the reading. Freezing and thawing of samples should be minimized, 3 freeze-thaw cycles showed no effect on the measured adiponectin concentration.

5. Interference

Hemoglobin, triglyceride and bilirubin in the sample do not interfere to a concentration of 5 mg/mL, 100 mg/mL and 100 µg/mL respectively. However, the use of hemolytic, lipemic or icteric samples should be validated by the user.

6. Sample dilution

• Dilution: 1:310 with Dilution Buffer DIL

• Dilute for example 300 µL Dilution Buffer DIL in PE-/PP-Tubes (application of a multi stepper is recommended in larger series), add 10 µL Serum- or Plasma (dilution: 1:31). Add 900 µL Dilution Buffer DIL in another PE-/PP-tube and 100 µL of the thoroughly mixed first dilution. After mixing, use 2×100 µL from this 1:310 diluted sample in the assay.

• Sample stability after dilution of the sample: maximum 1 hour at 20-25°C.

Reagent Preparation

Bring all reagents to room temperature (20 - 25°C) before use. Possible precipitations in the buffers have to be resolved before usage by mixing and / or warming. Reagents with different lot numbers cannot be mixed.

1. The Calibrators A – E is reconstituted with 750 µL Dilution Buffer DIL. After resuspension, the standard is diluted according to a gradient - Dilution Buffer DIL (Blank), A (2 ng/mL), B (10 ng/mL), C (30 ng/mL), D (70 ng/mL) and E (100 ng/mL), which are prepared for immediate use.
2. The Controls CTR1 and CTR2 are reconstituted with 500 µL Dilution Buffer DIL. After reconstitution dilute the Controls CTR1 and CTR2 with the Dilution Buffer DIL in the same ratio (1:310) as the sample.

Note: It is recommended to keep reconstituted reagents at room temperature for 15 minutes and then to mix them thoroughly but gently (no foam should result) with a Vortex mixer.

3. The required volume of Washing Buffer WB is prepared by 1:20 dilution of the provided 20-fold concentrate with Aqua dest.

Assay Procedure

Note

When performing the assay, Blank, Calibrators A-E, Controls CTR1, CTR2 and the samples should be pipette as fast as possible (e.g.<15 minutes). To avoid distortions due to differences in incubation times, Antibody Conjugate DET as well as the succeeding Substrate S should be added to the plate in the same order and in the same time interval as the samples. Stopping Solution STP should be added to the plate in the same order as Substrate S. All determinations (Blank, Calibrators A-E, Controls CTR1, CTR2 and samples) should be assayed in duplicate. For optimal results, accurate pipetting and adherence to the

protocol are recommended.

Incubation-Incubation at room temperature means: Incubation at 20 - 25°C. The Substrate S, stabilised H_2O_2-Tetramethylbencidine, is photosensitive–store and incubation in the dark.

Shaking-The incubation steps should be performed at mean rotation frequency of a particularly suitable microtiter plate shaker. We are recommending 350 rpm. Due to certain technical differences deviations may occur, in case the rotation frequency must be adjusted. Insufficient shaking may lead to inadequate mixing of the solutions and thereby to low optical densities, high variations and/ or false values, excessive shaking may result in high optical densities and/ or false values.

Washing-Proper washing is of basic importance for a secure, reliable and precise performance of the test. Incomplete washing is common and will adversely affect the test outcome. Possible consequences may be uncontrolled unspecific variations of measured optical densities, potentially leading to false results calculations of the examined samples. Effects like high background values or high variations may indicate washing problems. All washing must be performed with the provided Washing Buffer WB diluted to usage concentration. Washing volume per washing cycle and well must be 300 µL at least. The danger of handling with potentially infectious material must be taken into account.

When using an **automatic microtiter** plate washer, the respective instructions fur use must be carefully followed. Device adjustments, e.g. for plate geometry and the provided washing parameters, must be performed. Dispensing and aspirating manifold must not scratch the inside well surface. Provisions must be made that the remaining fluid volume of every aspiration step is minimized. Following the last aspiration step of each washing cycle, this could be controlled, and possible remaining fluid could then be removed, by inverting the plate and repeatedly tapping it dry on non-fuzzy absorbent tissue.

Manual washing is an adequate alternative option. Washing Buffer may be dispensed via a multistepper device, a multichannel pipette, or a squirt bottle. The fluid may be removed by dynamical swinging out the microtiter plate over a basin. If aspirating devices are used, care has to be taken that the inside well surface is not scratched. Subsequent to every single washing step, the remaining fluid should be removed by inverting the plate and repeatedly tapping it dry on non-fuzzy absorbent tissue.

Assay Step

1. Set Calibrators A-E, Sample SPE (1:310 diluted), Control CTR1 and CTR2 (1:310 diluted) wells on the pre-coated plate respectively, and then, records their positions. It is recommended to measure each standard and sample in duplicate.
2. Prepare Standards: Aliquot 100ul of Calibrators A-E, Control CTR1/CTR2 or samples into wells.
3. Incubate: Seal the plate with a cover and incubate at 20-25°C, 350 rpm for 1 hour.
4. Wash: Aspirate the contents of the wells, and wash plate 3 times with 300 µL Washing Buffer WB. Do not let the wells dry completely at any time.
5. Add 100ul Antibody-HRP-Conjugate DET into above wells. Add the solution at the bottom of each well without touching the sidewall, cover the plate and incubate at 20-25°C, 350 rpm for 30 minutes.
6. Wash: Aspirate the contents of the wells, and wash plate 3 times with 300 µL Washing Buffer WB. Do not let the wells dry completely at any time.
7. Add 100ul of Substrate Solution S into each well, cover the plate and incubate at 20-25°C in dark within 15 minutes.
8. Stop: Add 100ul Stopping Solution STP into each well.
9. Measure the absorbance within 30 min at 450 nm, with ≥ 590 nm as reference wavelength.

Quality Control

Good laboratory practice requires that controls are included in each assay. A statistically significant number of controls should be assayed to establish mean values and acceptable ranges to assure proper performance. The test results are only valid if the test has been performed following the instructions. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable federal, state or local standards/laws. All calibrators and kit controls must be found within the acceptable ranges as stated on the QC Certificate. If the criteria are not met, the run is not valid and should be repeated. Each laboratory should use known samples as further controls.

Quality criteria

For the evaluation of the assay it is required that the absorbance values of the blank should be below 0.25, and the absorbance of calibrator E should be above 1.00. Samples, which yield higher absorbance values than calibrator E, should be re-tested with a higher dilution.

Calculation

1. Calculate the mean absorbance value for the blank from the duplicated determination (well A1/A2).
2. Subtract the mean absorbance of the blank from the mean absorbance of all other samples and calibrators.
3. Plot the calibrator concentrations on the x-axis versus the mean value of the absorbance of the calibrators on the y-axis.
4. Recommendation: Calculation of the calibrator curve should be done by using a computer program, because the curve is in general (without respective transformation) not ideally described by linear regression. A higher-grade polynomial, or four parametric logistic (4-PL) curve fit or non-linear regression are usually suitable for the evaluation (as might be spline or point-to-point alignment in individual cases).
5. The Adiponectin concentration in ng/mL of the samples and controls CTR1 and CTR2 can be calculated by multiplication with the respective dilution factor.

Calibrator	A	B	C	D	E
ng/mL	2	10	30	70	100

Exemplary calculation of Adiponectin concentrations

Sample dilution: 1:310

Measured extinction of your sample 0.408

Measured extinction of the blank 0.008

Your measurement programm will calculate the Adiponectin concentration of the diluted sample automatically by using the difference of sample and blank for the calculation. You only have to determine the most suitable curve fit (here: polynomial 2nd degree).

In this exemplary case the following equation is solved by the program to calculate the adiponectin concentration in the sample:

$0.400 = -6 \times 10^{-5}x^2+0.0344x$

$11.89 = x$

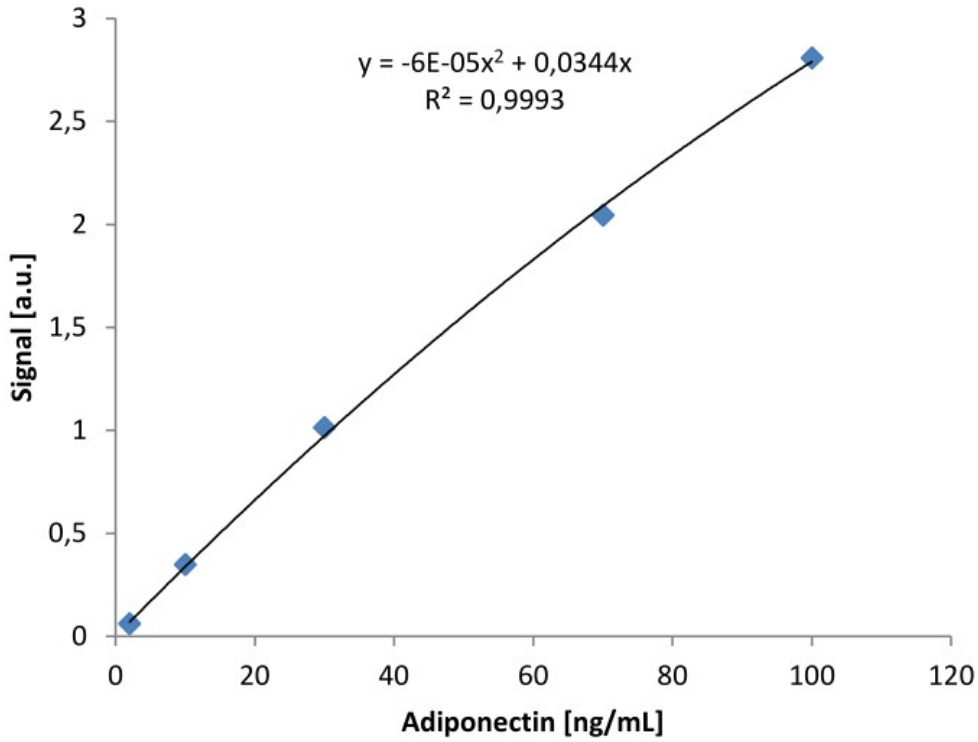
If the dilution factor (1:310) is taken into account the adiponectin concentration of the undiluted sample is

11.89 × 310 = 3685.9 ng/mL = 3.69 µg/mL

Typical Standard Curve

The following data is for demonstration only and cannot be used in place of data generation at the time of assay.

	Blank	A	B	C	D	E
ng/mL	0.0	2	10	30	70	100
$OD_{(450\text{-}620\ nm)}$	0.008	0.071	0.357	1.022	2.053	2.817



Note: The exemplary shown standard curve cannot be used for calculation of your test results. You have to establish a standard curve for each test you conduct!

Precision

Intra-Assay Variance & Accuracy

Intra assay variance and accuracy is exemplarily shown with two samples. The adiponectin concentration of these samples was repeatedly measured in one assay.

Recombinant adiponectin was diluted in dilution buffer and the adiponectin concentration of the dilution was measured repeatedly within one assay.

In both samples the variability is less than 5% and the deviation from the target value is <20%.

	Determinations [n]	Mean value [µg/L]	Standard deviation [µg/L]	CV [%]	Target Value [µg/L]
Sample 1	8	7.108	0.22	3.14	6
Sample 2	8	107.96	3.97	3.67	100

Inter-Assay Variance

Serum samples where repeatedly measured in independent assays of different lots. On average the coefficient of variation was 7.5% (SD 1.6).

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Mean [µg/mL]	4.72	5.25	8.36	5.45	22.29
CV [%]	8.16	8.14	6.93	8.05	7.30
n	99	68	62	174	62

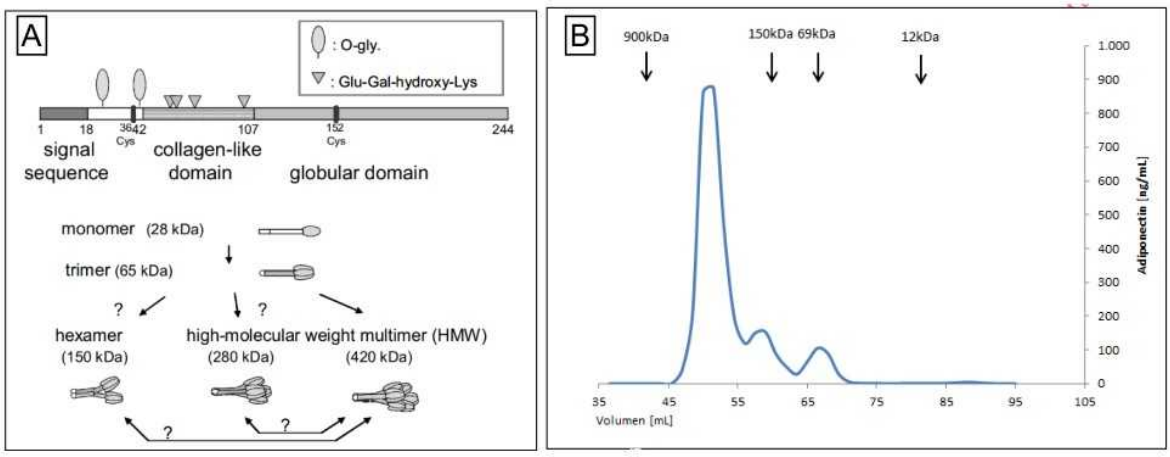
Sensitivity

The analytical sensitivity of the Human Adiponectin ELISA Kit was measured by the variability of the signal of the blank. Based on the two-fold standard deviation of the blank the mean analytical sensitivity is < 0.27 ng/mL (Range 0.094 to 0.59 ng/mL).

Specificity

Adiponectin exists in different oligomeric forms: the high, medium and low molecular weight form. Different numbers of the adiponectin monomer aggregate specifically to form a complex. In Figure A the five different forms of human adiponectin are shown schematically. In parallel the results of a size-exclusion chromatography of human serum measured with the Human Adiponectin ELISA Kit are shown.

The results shown in Figure B clearly demonstrate that the Human Adiponectin ELISA Kit detects all forms of Adiponectin present in human serum: the trimer at 65 kDa, the hexamer at 150 kDa and the high molecular weight forms of >280 kDa. The Human Adiponectin ELISA Kit therefore measures total Adiponectin.



Linearity

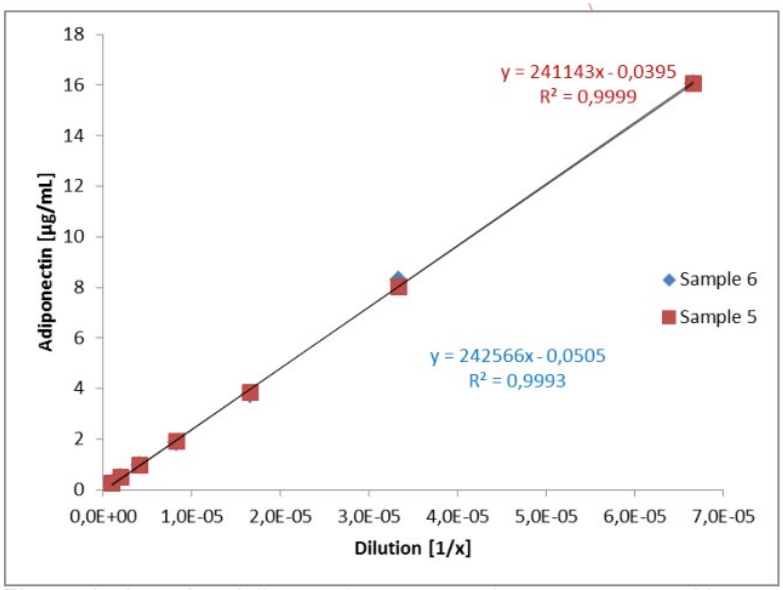
Linearity of sample dilution was tested by serial dilution (1:100 – 1:4000) of human serum samples and recalculation of the adiponectin content in comparison to the mean adiponectin concentration of all dilutions,

no diluted sample showed a deviation of >30%.

Table. Linearity. Human serum sample were diluted in DIL and adiponectin content was recalculated. Measurements results are shown in [mg/L]. No deviation of the mean >30% was detected.

µg/mL	Mean	1:100	1:200	1:400	1:800	1:1000	1:2000	1:4000
Sample 1	5.76	6.53	6.331	5.764	5.49	6.067	6.114	4.056
Sample 2	11.53	10.93	12.107	11.395	11.454	11.567	12.884	10.362
Sample 3	12.07	13.57	12.853	12.03	11.974	11.338	11.548	11.169
Sample 4	4.89	4.659	4.886	4.384	4.425	5.851	5.13	N/A

Additionally, dilutions of 1:1500 to 1:96000 were evaluated with two samples. In Figure below the results are shown and demonstrate that in the tested samples no effect of dilution could be detected on measured adiponectin concentrations. The deviation of the target concentration of each dilution was less than 30% in all dilutions.



Recovery

Trueness and traceability of the Human Adiponectin ELISA Kit was evaluated by recovery of recombinant adiponectin in human serum. The recovery of recombinant Adiponectin yielded in a serum matrix on average 110%.

Table. Recovery of recombinant human Adiponectin in Serum. Recombinant Adiponectin was added in different amounts to human serum. The Adiponectin content of the so enriched samples was measured and recovery in comparison to enriched buffer calculated.

Recombinant Adiponectin (ng/mL)	DIL (ng/mL)	Serum (ng/mL)	Recovery (%)
0	0	0.00778	—
75	87.92	95.71	109
37.5	51.84	59.98	116
18.75	26.55	26.7	101
9.375	13.35	15.15	113

Interferences

Interference of bilirubin and triglycerides was tested by adding different amounts of these substances to human serum containing adiponectin. For comparison the same amount of buffer without any substance was also added to the serum. Table below demonstrates that neither bilirubin nor triglycerides or haemoglobin exert any influence on the measurement of adiponectin in human serum.

Table. Interference. Serum samples were enriched with different amount of triglycerides, bilirubin or haemoglobin. The relative amount of adiponectin measured in comparison with native serum is shown here [%].

Triglyceride (100 mg/mL)	Bilirubin (100 µg/mL)	Hemoglobin (1 mg/mL)	Hemoglobin (5 mg/mL)
94	96	90	109
90	93	97	—
95	94	93	—

Precautions

1. For research and professional use only. The Creative Diagnostics kit is suitable only for in vitro use and not for internal use in humans and animals.
2. Follow strictly the test protocol. Use the valid version of the package insert provided with the kit. Be sure that everything has been understood. Creative Diagnostics will not be held responsible for any loss or damage (except as required by statute) howsoever caused, arising out of noncompliance with the instructions provided.
3. Do not use obviously damaged or microbial contaminated or spilled material.
4. This kit contains material of human and/or animal origin. Therefore all components and patient´s specimens should be treated as potentially infectious.
5. Appropriate precautions and good laboratory practices must be used in the storage, handling and disposal of the kit reagents. The disposal of the kit components must be made according to the local regulations.
6. Following components contain human serum: Controls CTR1 and CTR2, and Calibrators A-E. Source human serum for the control sera provided in this kit was tested and found non-reactive for Hepatitis-B surface antigen (HBsAg), Hepatitis C virus (HCV), and Human Immunodeficiency Virus 1 and 2 (HIV). No known methods can offer total security of the absence of infectious agents; therefore all components and patient´s specimens should be treated as potentially infectious.

Limitations

The Human Adiponectin ELISA Kit is based on monoclonal antibodies. Generally, this technique could be sensible to heterophilic antibodies or rheumatic factors in the sample. Their influence is reduced by assay design, but cannot be excluded completely.