Mercodia

Technical note, No: 34-0166, v.1.0

Total Insulin Northern Lights® MBeads Assay

10-1353-01

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384 well plate format

1. Material required but not provided

- Pipettes with appropriate volumes (automatic multichannel or repeating pipettes preferred for addition of MBeads Mix or other working solutions)
- Tubes, beakers, and cylinders for reagents preparation
- Vortex mixer
- Reagent reservoirs
- Microplate reader for chemiluminescence
- Microplate orbital shaker (recommended speed is 1350 cycles per minute)
- Magnetic bead-separator for 384 well plates
- Black 384 well plate
- Microplate washing device that also supports magnetic bead washing using a biomagnetic bead-separator

Note! If other plate format is being used (i.e., 96 well plate), a suited magnet for that specific plate is required

2. Automated washing

The Total Insulin Northern Lights® MBeads Assay protocol for 384 well plates, has been validated for automated washing using the “AquaMax 2000” Microplate washer (Molecular Devices). This device allows the use of the magnetic bead separator during the washing procedure.

Settings among washing devices might differ, keep in mind to adjust the settings according to the washing machine and the magnetic separator.

Example for the “AquaMax 2000” Microplate washer (Molecular Devices):

a) Aspirate (1 second, probe height 1.0 mm)
b) Dispense 70 µL (rate 5 of 5)
c) Soak 30 seconds
d) Repeat from #1 to #3 six times
e) Aspirate (1 second, probe height 1.0 mm)

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3. Plates

The Total Insulin Northern Lights MBeads Assay has been validated using 384 well plates produced by Corning. See table below.

Brand	Article No.	Name
Corning	3575	Corning® NBS™ (non-binding surface), 384 well microplates, black polystyrene, flat bottom, non-sterile

4. Reagents 1 x 384

Each Total Insulin Northern Lights MBeads Assay kit (10-1353-01) contains enough reagents for 500 datapoints. This would translate to more than 1x384 wells, sufficient for 184 samples and one calibrator curve in duplicate per plate. If needed, for larger series of assays, use pooled reagents from packages bearing identical lot numbers. The expiry date for the complete kit is stated on the outer label. The recommended storage temperature is 2–8°C.

Reagent	Amount	Volume	
MBeads Antibody Mouse monoclonal anti-insulin Storage after dilution: 2-8°C for 4 weeks	1 vial	2.5 mL	Preparation, See below
Calibrators 1, 2, 3, 4, 5, 6, 7 Recombinant human insulin Color coded yellow Concentration indicated on vial label	7 vials	1000 µL	Ready for Use
Calibrator 0 Color coded yellow	1 vial	5 mL	Ready for Use
Control Recombinant human insulin Color coded yellow Concentration indicated on vial label	1 vial	1000 µL	Ready for Use
Enzyme Conjugate 44X Peroxidase conjugated mouse monoclonal anti-insulin Storage after dilution: 2-8°C for 4 weeks	1 vial	1.0 mL	Preparation, see below
Assay Buffer Colorless solution	1 bottle	47 mL	Ready for Use

Wash Buffer 21X Storage after dilution: 2-8°C for 2 months	2 bottles	2x50 mL	Dilute 50 ml Wash Buffer 21X with 1000 mL redistilled water to make Wash Buffer 1X solution
Substrate Reagent A Colorless solution *Light sensitive!* *Note! The mixture of the two components is stable for 1 day at room temperature when protected from light.*	2 bottles	2x7 mL	Mix 1:1 with Substrate Reagent B to make substrate working solution
Substrate Reagent B Colorless solution *Note! Light sensitive!*	2 bottles	2x7 mL	Preparation, see Substrate Reagent A

5. Preparation of Enzyme Conjugate 1X solution

Prepare the needed volume of Enzyme Conjugate 1X solution by dilution of Enzyme Conjugate 44X in Assay Buffer according to the table below. Mix gently. Preferably use the Enzyme conjugate after preparation. If needed, store at 2–8°C and use within 4 weeks.

Number of wells	Enzyme Conjugate 44X	Assay Buffer
384 wells	170 µL	7.310 mL

6. Preparation of 0.2 % MBeads Antibody

Prepare needed volume of 0.2 % MBeads Antibody by diluting 1 % MBeads Antibody in Assay Buffer (1:5, respectively) according to the table below. Invert the vials with Assay buffer and MBeads Antibody five times. The MBeads Antibody contains beads that precipitate in the tube. The component must therefore be thoroughly mixed before use. For the same reason, pipetting must be carried out quickly and the pipette tips should not be prewetted. To make sure that all the beads are out of the pipette, pull the solution up and down 3-5 times. Mix gently. Store at 2–8°C and use within 4 weeks.

Number of wells	MBeads Antibody	Assay Buffer
384 wells	1.5 mL	6.0 mL

7. Preparation of MBeads Mix

The mixture of Enzyme Conjugate 1X and 0.2 % MBeads Antibody is called MBeads Mix. Prepare the MBeads Mix by mixing equal volumes of Enzyme Conjugate 1X and 0.2 % MBeads Antibody (1:1) according to the table below.

Note! Start by adding Enzyme Conjugate to a tube and thereafter the 0.2% MBeads Antibody solution. Make sure that the solution is homogenous before pipetting. The MBeads Antibody 0.2% solution contains beads that precipitate in the tube. Therefore, start by adding Enzyme conjugate 1X solution and then make sure the MBeads Antibody 0.2% solution is homogenous before adding it to the mixing tube. Pipetting must be carried out quickly and the pipette tips should not be prewetted.

To make sure that all the beads are out of the pipette, pull the solution up and down 3-5 times when adding MBeads Antibody. Vortex the MBeads Mix gently or invert the tube 5 times to ensure proper mix. Store at 2–8°C and use within 2 weeks.

Number of wells	Enzyme conjugate 1X	0.2 % MBeads Antibody
384 wells	7.4 mL	7.4 mL

8. Preparation of Wash Buffer 1X solution

Prepare the needed volume of wash buffer 1X solution by dilution of Wash Buffer 21X in redistilled water (1+20) according to the table below. Mix properly. Storage after dilution: 2–8°C for 2 months.

Number of plates	Wash Buffer 21X	Redistilled water
1 plate	50 mL	1000 mL

Note! If using an automatic washing machine, washing solution volumes might differ between washing machines. Priming volumes might be different between brands and models. See your washing machine specifications.

9. Preparation of substrate working solution

Prepare the needed volume of substrate working solution by mixing Substrate Reagent A with Substrate Reagent B (1:1) according to the table below. Mix gently and store at 2–8°C.

Note! The mixture of the two components is stable for 1 day at room temperature when protected from light.

Number of wells	Substrate Reagent A	Substrate Reagent B
384 wells	1 bottle	1 bottle

10. Test procedure for 384-well plate

All reagents and samples must be brought to room temperature before use.

1) Prepare wash buffer 1X solution (See section 8)
2) Prepare the Enzyme Conjugate solution 1X (See section 5) Light sensitive! To avoid the Enzyme being compromised, wrap foil around the tube for protection.
3) Prepare the 0.2 % MBeads Antibody (See section 6)
4) Pipette 5 µL each of Calibrators, controls, and samples into appropriate wells of a black 384-well assay plate. It is recommended to use a multi-channel pipette for this step.
5) Prepare MBeads Mix (See section 7) by mixing equal volumes of the prepared Enzyme Conjugate 1X solution and MBeads Antibody 0.2% solution.
6) Prepare substrate working solution by mixing equal volumes of Substrate Reagent A and Substrate Reagent B. (See section 9)
7) Invert the MBeads Mix five times and pour into a reagent reservoir.
8) Use a multi-channel pipette and transfer 30 µL of MBeads Mix to the wells of the assay plate. Shake the reservoir before pipetting to ensure a homogenous solution. Hold the pipette upright so that the beads do not settle on the inside of the tips, pipetting must be carried out quickly.
9) Incubate on an orbital plate shaker for 2 hour (1350 rpm) at room temperature (18–25°C).
10) Put the assay plate on the 384-well magnetic bead separator for 1 minute.
11) Wash the plate using automatic protocol. Wash 6 times with 70 µL wash buffer 1X solution per well and 30 seconds soak between cycles. Adjust the settings according to the washing machine and the magnetic separator to make sure that the washer tips go down far enough to soak up washing solution, but not too far down so that the washer soaks up beads. (See section 2)
12) After final wash, keep the magnet under the plate and invert the plate against absorbent paper carefully. Do not tap the plate.
13) Remove the magnet and add 25 µL substrate working solution into each well. Place plate on a shaker for approximately 5 seconds to ensure mixing.

14) Use a microplate reader for chemiluminescence. Incubate in the dark, preferably inside the reader, for 5 min at room temperature (18-25°C), without shaking.
15) Measure all visible light (glow) with an integration time of 0.5 second. No filter is needed. Use settings for a 384 well plate with flat bottom. Instrument settings should be used according to the manufacturer's instructions. Read within 5 minutes.
16) Apply curve fitting directly on raw data (RLU). Preferably use 5-parametric logistic regression with weighing using $1/Y^2$.

Note! Be careful not to contaminate the substrate working solution with enzyme conjugate solution.

11. Example of results

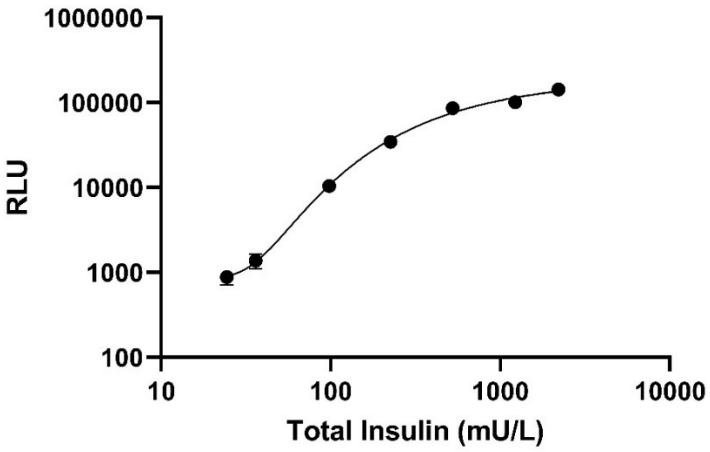
These values were obtained using BMG Labtech CLARIOstar with 0.5 s integration time, 3000 gain and 11.0 mm focal height.

Wells	Identity	RLU	Mean Conc. mU/L
1 A-B	*Calibrator 0*	*76/71*	
1 C-D	*Calibrator 1**	*938/1092*	
1 E-F	*Calibrator 2**	*1668/1551*	
1 G-H	*Calibrator 3**	*10560/11244*	
1 I-J	*Calibrator 4**	*32630/33307*	
1 K-L	*Calibrator 5**	*82756/88568*	
1 M-N	*Calibrator 6**	*98004/96318*	
1 O-P	*Calibrator 7**	*147817/147083*	
2 A-B	*Sample 1*	*2206/2442*	*48.7*
2 C-D	*Sample 2*	*25205/23785*	*172.5*
2 E-F	*Sample 3*	*95582/96565*	*732.5*
2 G-H	*Sample 4*	*24494/29910*	*180.1*

*Concentration stated on vial label.

12. Example of calibrator curve

A typical calibrator curve is shown here. Do not use this curve to determine actual assay results.



13. Precision and Accuracy

Samples and Control were analyzed in 4 replicates over 6 different occasions, on one kit lot, one instrument system and on two days by two laboratory technicians.

		Coefficient of variation	
Sample	Mean value mU/L	Repeatability %*	Within laboratory %**
Sample 1	49.4	12.5	15.3
Sample 2	184	17.6	20.6
Sample 3	605	16.8	23.2
Control	177	17.7	19.2

*Within assay variation

**Total assay variation

14. Parallellism

One human perifusion sample spiked with insulin to high concentrations within the measuring range were diluted 1/2 and 1/4. Mean recovery for parallelism is 85 % with precision between samples in the dilution series ≤ 16 %.

Three human perifusion samples with high insulin concentrations were diluted 1/8, 1/16 and 1/32. Mean recovery for parallelism is 101 % (94-112 %) with precision between samples in the dilution series ≤ 22 %.

Four mouse perifusion samples spiked with insulin to high concentrations within the measuring range were diluted 1/2 and 1/4. Mean recovery for parallelism is 95 % (83-106 %) with precision between samples in the dilution series ≤ 19 %.

One mouse perifusion sample with high insulin concentration was diluted 1/8, 1/16 and 1/32. Mean recovery for parallelism is 98 % with precision between samples in the dilution series ≤ 10 %.

15. Summary of protocol sheet

Summary of protocol sheet
Total Insulin Northern Lights MBeads Assay for 384 well plate

Prepare solutions	-	Wash Buffer 1X - Enzyme Conjugate solution 1X - 0.2 % MBeads Antibody - MBeads Mix
Pipette 5 µL in each well	-	Calibrators - Controls - Samples
Pipette MBeads Mix	-	Place 30 µL of the MBeads Mix in each well
Incubation	-	2 hours / 1350 rpm / room temperature (18-25°C)
Separate	-	Put the plate on the 384-well magnetic bead separator for 1 minute
Wash	-	Automatic (6 times with 70 µL per well and 30 seconds soak between cycles).
Dry up	-	Keep the magnet under the plate and invert the plate against absorbent paper carefully
Prepare solution	-	Substrate working solutions (reagents A+B)
Add substrate	-	Remove the magnet and add 25 µL substrate working solution into each well. Place plate on a shaker for approximately 5 seconds to ensure mixing.
Incubate	-	5 minutes at room temperature protected from light
Read chemiluminescence	-	0.5 s integration time (glow)

Other sample types: cell lysates and serum

The Total Insulin Northern Lights MBeads Assay is expected to react with human and mouse serum and cell lysates if their concentrations are in the measuring range. Cell lysates can be measured with and without acidic ethanol. It is recommended to keep the samples cooled after collection and store the samples at -20 °C until analysis.

Note! Serum samples usually have lower insulin concentration than the lowest calibrator and will not be measured in this assay.

Calibrator concentrations in different species and units

Calibrators	Human		Mouse	Rat
	mU/L	µg/L	µg/L	µg/L
Cal 1	23	1.0	1.47	0.90
Cal 2	33	1.43	2.11	1.29
Cal 3	110	4.78	7.04	4.29
Cal 4	242	10.5	15.5	9.44
Cal 5	527	22.9	33.7	20.6
Cal 6	1150	50	73.6	44.9
Cal 7	2500	108.7	160	97.5

Note! Calibrators may differ from lot to lot. Concentration for each calibrator is stated on the vial label. These values are only examples.

Note! Conversions should be made after reading the plate.

Cross-reactivity with mouse and rat c-peptide and proinsulin

The following cross reactions have been found:

Mouse C-peptide I	< 0.001 %
Mouse C-peptide II	< 0.0007 %
Mouse Proinsulin II	45 %
Rat C-peptide II	< 0.4 %
Rat C-peptide II	< 0.001 %
Rat Proinsulin I	3 %

For defining cross-reactivity we evaluated samples using the following concentrations:

Molecule	Cross-reaction	Concentrations tested	
		pmol/L	µg/L
Human C-peptide	n.d.	70000 – 790909	211 – 2389
Human Proinsulin	59%	201 – 604	1.9 – 5.7
Mouse Insulin	68%	3091 – 24726	17.94 – 143.5
Mouse C-peptide I	n.d.	2002300 – 16018402	6250 – 50000
Mouse C-peptide II	n.d.	2500000 – 20000000	7834 – 62668
Mouse Proinsulin II	45%	62 – 493	0.6 – 4.7
Rat Insulin	112%	489 – 1958	2.84 – 11.361
Rat C-peptide I	<0.4%	1219 – 32909	4.0 – 107
Rat C-peptide II	n.d.	1976928 – 15815428	6250 – 50000
Rat Proinsulin I	3%	129803 – 1038422	1250 – 10000

Note! Highlighted units were used for calculating cross-reactivity. For further questions contact: tech-support@mercodia.com.

Conversion factor for rat samples

1 mU/L = 0.039 µg/L

For converting mU/L to µg/L, divide the value in mU/L by 25.76. This number corresponds to the value acquired by multiplying 23x1.12; 23 being the number of mU/L in 1 µg/L and 1.12 the % of cross reactivity to Mouse Insulin (see cross reactivity in section above).

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