MANUAL FOR THE ASSESSMENT OF LOW SPERM COUNTS

AND

AZOOSPERMIA

Leja 100 micron 2 chamber slide
and
Leja 20 micron 2 chamber slide

General Information Leja® Standard Count counting chambers

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Leja is certified ISO 9001:2000

Leja slides are in vitro diagnostic devices to be used by an authorized person.

Leja slides are designed for microscopic quantitative evaluation of cells in suspension, like blood and semen. Leja slides are not made for self-testing. Authorized, qualified persons should handle Leja slides.

Leja slides should be stored in a closed box, protected from sunlight, and at room temperature. The shelf life of Leja slides is years, but a haze can form inside the chamber. Filling will wash this away and does not affect the functioning of the chamber.

Body fluids can be infectious. Do not touch the slides at the filling openings, and only touch the slide at the outer left and right edges. Prevent spilling of body fluids.
Leja slides are made of glass. Glass is breakable and sharp edges will be formed after breaking.

Handle used Leja slides as infectious waste and discard used Leja slides according to the local instructions for the handling of infectious waste.

General description.

The Leja slides are to be used for quantitative analysis of cells and particles in suspension. The slide has to be used with a microscope. The microscope can be integrated in a Computer Assisted Semen Analysis (CASA) system. They are mostly used for the analysis of semen. The Leja slides with chamber heights of 12 and 20 microns are suitable for the analysis of motile spermatozoa in combination with a 10x or 20x objective lenses. The Leja slide with a chamber height of 100 microns is made for the analysis of small numbers of cells. A separate instruction manual is available for the assessment of low numbers (0-8.000/ml and 8.000 – 1.10^6/ml): manual 20 and 100 micron Leja slides.

The Leja slide is made of a standard microscopic object slide with the dimensions of 75x25x1 mm. It is made of float glass and the dimensions of 75 x 25 mm are achieved by sawing, not by cutting and breaking. The sawed, blunt edges prevent damage to the skin of the user.
On top of the Leja chamber is a cover slip. The dimensions of the cover slip of the Leja 4 chamber slide are 32x 21x 0,7 mm.
Both glass plates are washed and coated. Clean glass has a very reactive surface; the coating prevents sticking of the cells to the glass surface. The coating prevents the formation of air bubbles during the filling process. However, small particles suspended in the semen sample can provoke the formation of air bubbles.

The particulars of the Leja slides are printed at the left and right sides of the object slide.

Leja slides are available with different chamber numbers and different chamber heights. There are slides with 2, 4 and 8 chambers and chamber heights are available in 12, 20 and 100 microns. The content of the chambers varies between 1 µl and 25 µl. The content of the chamber is indicated on the slide. The effective surface of the chamber to be used for analytical purposes varies between 20 mm^2 (eight chamber slide), 66 mm^2 (4 chamber slide), and 250 mm^2 (100 micron chamber height, two chamber slide).

The two glass plates of the Leja slide are at a well-defined distance from each other. A resin is printed on the object slide. In this resin spacers of a well-defined diameter are present. A cover slip is placed on the printed pattern and the two glass plates are pressed together in such a way that the two plates stay parallel**.** The spacers define the distance between the two glass plates. During this process the resin is cured. The production of the Leja slides takes place in a clean room, preventing the settlement of dust particles inside the chamber during the production process.

Patents protect the production process and the chamber designs.

Chamber height

The tolerance of the chamber height is maximum ± 5%. The 100-micron chamber has a chamber height of 100 ± 5 micron. The actual average height, the upper and lower limits and the 95% confidence interval of each lot is published at our web side.

Segre-Silberberg effect compensation factor

In principle, all capillary filled slides are affected by this phenomenon. It causes transport of cells to the filling front during the flow of the sample into the chamber. The Segre-Silberberg effect causes underestimation of the concentrations assessed in the central area of the analysis chamber. The dimension of the correction factor of the Segre-Silberberg effect depends on many variables, like the development of a full Poiseuille flow, chamber height, surface properties of the counting chamber, surface tension, flow velocity and viscosity of the sample. For sperm cells suspended in watery solutions the compensation factor will be 1.30. For very viscous samples the correction factor will be near 1.00. Since all variables are kept constant the only variable that affects the dimension of the Segre Silberberg effect is the viscosity of the sample. Filling time and viscosity are closely associated. By measuring the filling time of the Leja chamber with a capillary length of 21 mm, the Segre Silberberg compensation factor for that specific sample is known.

Due to the chamber height of 100 microns, the Segre Silberberg effect is negligible.

Quality control

1. Chamber height.

After the production process each slide must pass quality control. Printing quality and resin tracks are checked visually. Chamber height is checked with two methods. 1) All chambers are checked with interference patterns of monochromatic light. 2) Out of each box of 25 slides 3 slides are tested with a specific device, specially constructed for Leja, which measures the chamber height in microns. This device works according to the principle of interference of the complete spectrum of visible light. The results of the assessments of each lot are published at the website of Leja.

2. Toxicity.

Each lot is tested for toxicity with diluted boar semen; boar semen is much more sensitive to toxic effects than human spermatozoa. The motility of the spermatozoa is tested in the central area (at least 0.8 mm from the resin border). The motility may decrease with a maximum of 10 % within a test period of 6 minutes.

The results of the toxicity assay are published at the website of Leja. 100 micron chambers are not tested for toxicity; sharpness of vision of 10x and 20x objective lenses is much less than 100 micron.

3. Air bubble formation.

Each lot is tested for air bubble formation during the filling process. Each day slides are randomly selected and filled with water. Lots pass quality control when no air bubble formation is observed.

Waste handling.

Unused Leja chambers can be discarded as household waste. Leja chambers filled with human bodily fluids have to be treated as infectious and should be treated accordingly. Follow the instructions of local health authorities how to treat used, filled Leja chambers.

The assessment of low sperm counts and azoospermia

Follow the instructions of the *WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction* (see reference list) for collection and handling and of human semen. Human semen can be infectious. Always treat it as infectious material.

Purpose of procedure

This manual describes a method for the assessment of low sperm counts (0 – 8.000/ml) with Leja 2 chamber 100 microns slides and the assessment of sperm concentrations 8.000/ml - 1.10^6/ml with a Leja 2 chamber 20 micron slides.

Materials needed

- Phase contrast microscope with 10x and 20x objective lenses
- 20 µ 2 chamber slide
- 100 µ 2 chamber Leja slide
- Hot plate 60°C or formaldehyde
- Pipettes to pipette 10 µl and 25 µl
- Stopwatch
- Cell counter
- Humidified box

Variables and formulas

Radius	=	diameter / 2
π	=	pi: 3,14
µ	=	micron
VM_x	=	volume one microscopic field
VM_{10}	=	volume one microscopic field using10x objective lens = radius * radius * chamber height * 3.14
VM_{20}	=	volume one microscopic field using 20 x objective lens = radius * radius * chamber height * 3.14
FT	=	filling time in seconds
TC_1	=	total number of counted cells in the first round
TC_2	=	total number of counted cells in the second round
AF_1	=	Number of assessed field in the first round
AF_2	=	Number of assessed field in the second round
VL_1	=	Assessed volume first round = AF_1 * VM
VL_2	=	Assessed volume second round = AF_2 * VM
C_{i1}	=	First initial concentration = TC_1 / VL_1 (10^6 / ml)
C_{i2}	=	Second initial concentration = TC_2 / VL_2 (10^6 / ml)
S_x	=	Segre Silberberg compensation factor, see Appendix 2
C_{t1}	=	exact concentration first round (corrected for the Segre Silberberg effect)
C_{t2}	=	exact concentration second round (corrected for the Segre Silberberg effect)
C_f	=	final exact concentration (C_{t1} + C_{t2}) / 2

Calibration of your microscope.

- Measure the diameter of the microscopic field with a stage micrometer. The radius is half the diameter. In many microscopes without in between lenses the diameter of the microscopic field using a 10x objective lens will be 2.000 µ. The radius will be 1.000 µ. In many microscopes, using a 20x objective lens the diameter of the microscopic field will be 950 µ and the radius will be 475 µ.

- Take the radius in microns. The chamber height is 20 µ. The volume of one field is $radius^2 * 20 * \pi$. One will get a figure with 8 digits. Since 1 nl = 10^6 μ^3, one has to divide the figure by 10^6 to get nl.

- VM_{10} = the volume in nl of one microscopic field using a 10x objective lens

- VM_{20} = the volume in nl of one microscopic field using a 20x objective lens

- In these examples using a 10x objective lens and a Leja 20 µ chamber, the volume of one microscopic field will be 62,8 nl = $1.000^2 * 20 * 3,14 / 10^6$ = 62,8 nl (if the radius is 1000m).

- If for example the average number of spermatozoa per field is 5, the concentration will be 5 / 62,8 = 0,080 /nl sperm cells or $0,080.10^6$ /ml (80.000 sperm cells per ml).

- Using a 20x objective lens the volume of one microscopic field will be 14,2 nl (if the radius is 475 µ)

- If for example, the average number of sperm cells is 7 per microscopic field, the concentration will be 7 / 14,2 = 0,49 / nl sperm cells or $0,49.10^6$/ml (= 490.000 sperm cells per ml)

Procedure

- Let semen liquefy
- Mix the sample carefully, prevent foaming
- Load a chamber of the Leja 2 chamber 20 µ slide
- Take 10 µl of the well mixed semen
- Keep the stopwatch ready
- Place the tip of the pipette at the loading area of a 2 chamber 20 µ slide
- Push the piston of the pipette and the stopwatch at the same time
- Push the stopwatch again as soon as the semen reaches the air outlet.
- Take away the surplus of semen at the inlet
- Note the filling time = **FT**

  One has to use more semen than the content of the chamber; otherwise it will be not possible to properly assess the loading time.

- Place the chamber on the microscope stage
- Study the content of the slide.
- Using a 10x objective lens: If in total 5 cells or less in 10 fields are counted, the concentration will be lower than 8.000 sperm cells/ml:

$$\frac{5}{10 \cdot 62.8} = 0.008 \frac{sperm\ cells}{nl}$$

  (the volume of 62,8 nl is the volume of one microscopic field in this example).

If the concentration found to be higher than 10^6 cells/ml, continue the assessment of sperm concentration according standard methods (see following page for procedure or see the manual for semen assessment using 20 micron slides).

If the concentration found to be less than 10^6 cells/ml, continue the assessment of sperm concentration with a 100 micron chamber (see following chapter or manual for semen assessment using 100 micron slides).

- If after the initial assessments the concentration will be between 8000 and 1.10^6/ml, it is advised to choose at random five fields or more and to count all the spermatozoa present in these fields. It is advised to count at least 200 cells, if possible.

- Note the number of initial counted cells = $\mathbf{TC_1}$

- Note the number of assessed fields in the first round = $\mathbf{AF_1}$

- Calculate assessed volume $\mathbf{VL_1 = AF_1 * VM_x}$

- Calculate the first initial concentration $\mathbf{C_{i1} = TC_1 / VL_1}$ **(10^6 / ml)**

- Correction for the SS effect. The filling time of the Leja 20 micron 2 chamber slide was measured (**FT**)

- Go to Appendix 2

- Read in the first column the filling time (which you measured already) and in the second column the correction factor of the SS effect = $\mathbf{S_x}$

- Get the first exact concentration $\mathbf{C_{t1} = C_{i1} * S_x}$

- Perform a second assessment. Load the second chamber of the slide. Measure the filling time $\mathbf{FT_2}$ and get $\mathbf{TC_2}$, the total number of counted cells in the second round, and calculate the second initial concentration $\mathbf{C_{i2}}$ and the second true concentration $\mathbf{C_{t2}}$. Note the number of counted cells in the second round $\mathbf{TC_2}$.

- Take $\mathbf{2 * \sqrt{(TC_1 + TC_2)}}$, two times the square root of the total number of assessed cells

- Take $\mathbf{|TC_1 - TC_2|}$, the absolute value of the difference of the two numbers of assessed cells

- If $\mathbf{|TC_1 - TC_2|} \leq 2 * \sqrt{(TC_1 + TC_2)}$ the sampling taking and the assessments have resulted in two observations within the limits of 95% confidence interval the assessments are accepted.

- If $\mathbf{|TC_1 - TC_2| > 2 * \sqrt{(TC_1 + TC_2)}}$, the assessments are rejected and one has to start over completely.

- If the two assessments are accepted, calculate the exact final concentration $\mathbf{C_f}$ by taken the average value of $\mathbf{C_{t1}}$ and $\mathbf{C_{t2}}$; $\mathbf{C_f = (C_{t1} + C_{t2}) / 2}$

Assessment of low sperm concentrations with a Leja 2 chamber 100 µ slide

Procedure

- The Leja 2 chamber 100µ slide cannot be used for the assessment of motile sperms. One has to immobilize the sperms before the assessment. One can pipette a small amount of 36% formaldehyde (5 µl) to the semen sample or one can place the loaded chamber for 30 seconds on a hot plate (60°C).
- Load chamber A of the Leja 2 chamber slide with 25µl semen (formaldehyde treated) or place the slide on the hot plate.
- 100 micron is out of range of the sharpness of vision of both 10x and 20x objective lenses. Let the cells settle to the bottom for 5 – 10 minutes. To prevent drying out place the slide in the humidified box.
- Place the Leja chamber on the microscope stage
- Assess the number of sperms cells present in the whole chamber. Using a phase contrast microscope it will be very well possible to search the complete chamber and to assess the number of sperms present in the whole chamber.
- Go to table 1 and find the concentration and the 95% or 99% confidence intervals for the number of cells which were detected
- Load chamber B and repeat the assessment
- If the 95% confidence intervals overlap the assessments are accepted. Calculate the average value.

If more than 200 cells are present it is advised to stop the procedure and to use the Leja 20 micron chamber to assess the sperm concentration.

Example 1

If after meticulous searching the whole 100 micron chamber 0 cells are seen the concentration is

$0,000.10^{6}$ ml^{-1}

(CI 95%: $0,000000.10^{6}$ - $0.000120.10^{6}$; CI 99%: $0,000000.10^{6}$- $0,000184.10^{6}$);

Example 2

If 5 sperm cells are seen in a whole 100 micron chamber the concentration is

$0,000200.10^{6}$ ml^{-1}

(CI 95%: $0,000065.10^{6}$ – $0,000467.10^{6}$; CI 99%: $0.,000043.10^{6}$ – $0,000566.10^{6}$).

Appendix 1. Correction for Segre Silberberg effect

Filling time in seconds	S_x	***Filling time*** in seconds	S_x
2,0	1,32	9,0	1,09
2,1	1,31	10,0	1,08
2,2	1,30	11,0	1,08
2,3	1,29	12,0	1,07
2,4	1,28	13,0	1,06
2,5	1,27	14,0	1,06
2,6	1,26	15,0	1,06
2,8	1,25	16,0	1,05
2,9	1,24	17,0	1,05
3,2	1,23	18,0	1,05
3,4	1,22	19,0	1,04
3,6	1,21	20,0	1,04
3,8	1,20	21,0	1,04
4,0	1,19	22,0	1,04
4,2	1,18	23,0	1,04
4,5	1,17	24,0	1,04
5,0	1,16	25,0	1,03
5,3	1,15	30,0	1,03
5,5	1,14	60,0	1,01
6,0	1,13	120,0	1,01
7,0	1,11	180,0	1,00
8,0	1,10	240,0	1,00

In this table the relationship between *filling time* and S_x, the compensation factor of the Segre Silberberg effect is depicted. The figures are only valid for Leja chambers with a capillary length of 21 mm and a chamber height of 20 µm and for watery solutions, like semen or plasma. Filling time 2,2 seconds equals the filling time of water (viscosity 1 cP).
The slides with a chamber depth 100 micron do not show a Segre Silberberg effect due to the proportion on the chamber height and the diameter of the head of the spermatozoa.

Appendix 2. Observed numbers, concentration and 95% and 99% confidence intervals.

X = number of cells counted
Concentration = 10^6 cells ml^{-1}
CI = Confidence interval in cells 10^6 ml^{-1}

X	Concentration 10^6 ml^{-1}	CI 95% lower	CI 95% upper	CI 99% lower	CI 99% upper
0	0,000000	0,000000	0,000120	0,000000	0,000184
1	0,000040	0,000001	0,000223	0,000000	0,000240
2	0,000080	0,000010	0,000289	0,000000	0,000371
3	0,000120	0,000025	0,000351	0,000014	0,000439
4	0,000160	0,000044	0,000410	0,000027	0,000504
5	0,000200	0,000065	0,000467	0,000043	0,000566
6	0,000240	0,000088	0,000522	0,000062	0,000626
7	0,000280	0,000112	0,000577	0,000082	0,000685
8	0,000320	0,000138	0,000630	0,000103	0,000743
9	0,000360	0,000165	0,000683	0,000125	0,000800
10	0,000400	0,000192	0,000736	0,000149	0,000856
11	0,000440	0,000220	0,000787	0,000173	0,000911
12	0,000480	0,000248	0,000838	0,000198	0,000966
13	0,000520	0,000277	0,000889	0,000223	0,001020
14	0,000560	0,000306	0,000940	0,000249	0,001074
15	0,000600	0,000336	0,000990	0,000276	0,001126
16	0,000640	0,000366	0,001039	0,000303	0,001179
17	0,000680	0,000396	0,001089	0,000330	0,001232
18	0,000720	0,000427	0,001138	0,000358	0,001284
19	0,000760	0,000458	0,001187	0,000386	0,001335
20	0,000800	0,000489	0,001236	0,000414	0,001387
21	0,000840	0,000520	0,001284	0,000443	0,001438
22	0,000880	0,000552	0,001332	0,000472	0,001489
23	0,000920	0,000583	0,001380	0,000501	0,001539
24	0,000960	0,000615	0,001428	0,000530	0,001590
25	0,001000	0,000647	0,001476	0,000560	0,001640
26	0,001040	0,000679	0,001524	0,000590	0,001690
27	0,001080	0,000712	0,001571	0,000620	0,001740
28	0,001120	0,000744	0,001619	0,000650	0,001790
29	0,001160	0,000777	0,001666	0,000680	0,001839
30	0,001200	0,000810	0,001713	0,000711	0,001888
31	0,001240	0,000842	0,001760	0,000741	0,001938
32	0,001280	0,000876	0,001807	0,000772	0,001987
33	0,001320	0,000909	0,001854	0,000803	0,002036
34	0,001360	0,000942	0,001900	0,000834	0,002084
35	0,001400	0,000975	0,001947	0,000866	0,002133
36	0,001440	0,001008	0,001994	0,000897	0,002182
37	0,001480	0,001042	0,002040	0,000928	0,002230
38	0,001520	0,001076	0,002086	0,000960	0,002278
39	0,001560	0,001109	0,002132	0,000992	0,002326
40	0,001600	0,001143	0,002179	0,001024	0,002374
41	0,001640	0,001177	0,002225	0,001055	0,002422

X	Concentration 10^6 ml^{-1}	CI 95% lower	CI 95% upper	CI 99% lower	CI 99% upper
42	0,001680	0,001211	0,002271	0,001087	0,002467
43	0,001720	0,001245	0,002317	0,001120	0,002518
44	0,001760	0,001279	0,002363	0,001152	0,002566
45	0,001800	0,001313	0,002408	0,001184	0,002614
46	0,001840	0,001347	0,002454	0,001216	0,002661
47	0,001880	0,001381	0,002500	0,001249	0,002709
48	0,001920	0,001416	0,002546	0,001281	0,002756
49	0,001960	0,001450	0,002591	0,001314	0,002803
50	0,002000	0,001484	0,002637	0,001346	0,002851
51	0,002040	0,001519	0,002682	0,001379	0,002898
52	0,002080	0,001554	0,002728	0,001412	0,002945
53	0,002120	0,001588	0,002773	0,001445	0,002992
54	0,002160	0,001623	0,002818	0,001478	0,003039
55	0,002200	0,001657	0,002864	0,001511	0,003086
56	0,002240	0,001692	0,002909	0,001544	0,003133
57	0,002280	0,001727	0,002954	0,001577	0,003180
58	0,002320	0,001762	0,002999	0,001610	0,003226
59	0,002360	0,001796	0,003044	0,001644	0,003273
60	0,002400	0,001832	0,003089	0,001677	0,003320
61	0,002440	0,001866	0,003134	0,001710	0,003366
62	0,002480	0,001901	0,003179	0,001744	0,003413
63	0,002520	0,001936	0,003224	0,001777	0,003459
64	0,002560	0,001972	0,003269	0,001811	0,003506
65	0,002600	0,002007	0,003314	0,001844	0,003552
66	0,002640	0,002042	0,003359	0,001878	0,003598
67	0,002680	0,002077	0,003404	0,001912	0,003644
68	0,002720	0,002112	0,003448	0,001946	0,003691
69	0,002760	0,002148	0,003492	0,001979	0,003737
70	0,002800	0,002183	0,003538	0,002013	0,003783
71	0,002840	0,002218	0,003582	0,002047	0,003829
72	0,002880	0,002254	0,003627	0,002081	0,003875
73	0,002920	0,002289	0,003672	0,002115	0,003921
74	0,002960	0,002324	0,003716	0,002149	0,003967
75	0,003000	0,002360	0,003760	0,002183	0,004013
76	0,003040	0,002395	0,003805	0,002217	0,004059
77	0,003080	0,002431	0,003850	0,002251	0,004105
78	0,003120	0,002466	0,003894	0,002285	0,004151
79	0,003160	0,002502	0,003938	0,002319	0,004196
80	0,003200	0,002537	0,003983	0,002354	0,004242
81	0,003240	0,002573	0,004027	0,002388	0,004288
82	0,003280	0,002609	0,004071	0,002422	0,004334
83	0,003320	0,002644	0,004116	0,002456	0,004379
84	0,003360	0,002680	0,004160	0,002491	0,004425
85	0,003400	0,002716	0,004204	0,002525	0,004470
86	0,003440	0,002750	0,004248	0,002560	0,004516
87	0,003480	0,002787	0,004292	0,002594	0,004562
88	0,003520	0,002823	0,004337	0,002629	0,004607
89	0,003560	0,002859	0,004381	0,002663	0,004652
90	0,003600	0,002895	0,004425	0,002698	0,004698

X	Concentration 10^6 ml^{-1}	CI 95% lower	CI 95% upper	CI 99% lower	CI 99% upper
91	0,003640	0,002931	0,004469	0,002732	0,004743
92	0,003680	0,002967	0,004513	0,002767	0,004788
93	0,003720	0,003002	0,004557	0,002802	0,004834
94	0,003760	0,003038	0,004601	0,002836	0,004879
95	0,003800	0,003074	0,004645	0,002871	0,004924
96	0,003840	0,003110	0,004689	0,002906	0,004970
97	0,003880	0,003146	0,004733	0,002940	0,005015
98	0,003920	0,003182	0,004777	0,002975	0,005052
99	0,003960	0,003218	0,004821	0,003010	0,005105
100	0,004000	0,003254	0,004865	0,003045	0,005150
101	0,004040	0,003291	0,004909	0,003080	0,005196
102	0,004080	0,003327	0,004953	0,003114	0,005241
103	0,004120	0,003363	0,004997	0,003150	0,005286
104	0,004160	0,003399	0,005040	0,003184	0,005331
105	0,004200	0,003435	0,005084	0,003219	0,005376
106	0,004240	0,003471	0,005128	0,003254	0,005421
107	0,004280	0,003508	0,005172	0,003289	0,005466
108	0,004320	0,003544	0,005216	0,003324	0,005511
109	0,004360	0,003580	0,005260	0,003360	0,005556
110	0,004400	0,003616	0,005303	0,003394	0,005600
111	0,004440	0,003652	0,005347	0,003430	0,005645
112	0,004480	0,003689	0,005390	0,003465	0,005690
113	0,004520	0,003725	0,005434	0,003500	0,005735
114	0,004560	0,003762	0,005478	0,003535	0,005780
115	0,004600	0,003798	0,005522	0,003570	0,005825
116	0,004640	0,003834	0,005565	0,003606	0,005870
117	0,004680	0,003870	0,005609	0,003641	0,005914
118	0,004720	0,003907	0,005652	0,003676	0,005959
119	0,004760	0,003943	0,005696	0,003711	0,006004
120	0,004800	0,003980	0,005740	0,003746	0,006048
121	0,004840	0,004016	0,005783	0,003782	0,006093
122	0,004880	0,004052	0,005827	0,003817	0,006138
123	0,004920	0,004089	0,005870	0,003852	0,006182
124	0,004960	0,004126	0,005914	0,003888	0,006227
125	0,005000	0,004162	0,005957	0,003923	0,006272
126	0,005040	0,004198	0,006001	0,003959	0,006316
127	0,005080	0,004235	0,006044	0,003994	0,006361
128	0,005120	0,004272	0,006088	0,004030	0,006405
129	0,005160	0,004308	0,006131	0,004065	0,006450
130	0,005200	0,004344	0,006174	0,004100	0,006494
131	0,005240	0,004381	0,006218	0,004136	0,006539
132	0,005280	0,004418	0,006262	0,004171	0,006583
133	0,005320	0,004454	0,006305	0,004207	0,006628
134	0,005360	0,004491	0,006348	0,004242	0,006672
135	0,005400	0,004528	0,006392	0,004278	0,006716
136	0,005440	0,004564	0,006435	0,004314	0,006761
137	0,005480	0,004601	0,007678	0,004349	0,006805
138	0,005520	0,004638	0,006522	0,004385	0,006850
139	0,005560	0,004674	0,006565	0,004420	0,006894

X	Concentration 10^6 ml^{-1}	CI 95% lower	CI 95% upper	CI 99% lower	CI 99% upper
140	0,005600	0,004711	0,006608	0,004456	0,006938
141	0,005640	0,004748	0,006652	0,004492	0,006983
142	0,005680	0,004784	0,006695	0,004527	0,007027
143	0,005720	0,004821	0,006738	0,004563	0,007071
144	0,005760	0,004858	0,006781	0,004599	0,007116
145	0,005800	0,004894	0,006825	0,004634	0,007160
146	0,005840	0,004931	0,006868	0,004670	0,007204
147	0,005880	0,004968	0,006911	0,004706	0,007248
148	0,005920	0,005005	0,006954	0,004742	0,007293
149	0,005960	0,005042	0,006998	0,004778	0,007337
150	0,006000	0,005078	0,007041	0,004813	0,007381
151	0,006040	0,005115	0,007084	0,004849	0,007425
152	0,006080	0,005152	0,007127	0,004885	0,007470
153	0,006120	0,005189	0,007170	0,004921	0,007514
154	0,006160	0,005226	0,007213	0,004956	0,007558
155	0,006200	0,005262	0,007256	0,004992	0,007602
156	0,006240	0,005299	0,007300	0,005028	0,007646
157	0,006280	0,005336	0,007343	0,005064	0,007690
158	0,006320	0,005373	0,007386	0,005100	0,007734
159	0,006360	0,005410	0,007429	0,005136	0,007778
160	0,006400	0,005447	0,007472	0,005172	0,007822
161	0,006440	0,005484	0,007515	0,005208	0,007866
162	0,006480	0,005520	0,007558	0,005244	0,007910
163	0,006520	0,005558	0,007601	0,005280	0,007954
164	0,006560	0,005594	0,007644	0,005316	0,007998
165	0,006600	0,005631	0,007688	0,005352	0,008042
166	0,006640	0,005668	0,007730	0,005388	0,008086
167	0,006680	0,005705	0,007774	0,005424	0,008130
168	0,006720	0,005742	0,007816	0,005460	0,008174
169	0,006760	0,005779	0,007860	0,005496	0,008218
170	0,006800	0,005816	0,007902	0,005532	0,008262
171	0,006840	0,005853	0,007946	0,005568	0,008306
172	0,006880	0,005890	0,007988	0,005604	0,008350
173	0,006920	0,005927	0,008032	0,005640	0,008394
174	0,006960	0,005964	0,008074	0,005676	0,008438
175	0,007000	0,006001	0,008118	0,005712	0,008482
176	0,007040	0,006038	0,008160	0,005748	0,008526
177	0,007080	0,006075	0,008203	0,005784	0,008570
178	0,007120	0,006112	0,008246	0,005820	0,008614
179	0,007160	0,006150	0,008289	0,005857	0,008657
180	0,007200	0,006186	0,008332	0,005893	0,008701
181	0,007240	0,006224	0,008375	0,005929	0,008745
182	0,007280	0,006261	0,008418	0,005965	0,008789
183	0,007320	0,006298	0,008461	0,006001	0,008833
184	0,007360	0,006335	0,008504	0,006038	0,008876
185	0,007400	0,006372	0,008546	0,006074	0,008920
186	0,007440	0,006409	0,008590	0,006110	0,008964
187	0,007480	0,006446	0,008632	0,006146	0,009008
188	0,007520	0,006484	0,008675	0,006182	0,009052

X	Concentration 10^6 ml^{-1}	CI 95% lower	CI 95% upper	CI 99% lower	CI 99% upper
189	0,007560	0,006520	0,008718	0,006219	0,009095
190	0,007600	0,006558	0,008761	0,006255	0,009139
191	0,007640	0,006595	0,008804	0,006291	0,009183
192	0,007680	0,006632	0,008846	0,006328	0,009226
193	0,007720	0,006669	0,008889	0,006364	0,009270
194	0,007760	0,006706	0,008932	0,006400	0,009314
195	0,007800	0,006758	0,008975	0,006436	0,009358
196	0,007840	0,006781	0,009018	0,006473	0,009401
197	0,007880	0,006818	0,009060	0,006509	0,009445
198	0,007920	0,006855	0,009103	0,006545	0,009488
199	0,007960	0,006892	0,009146	0,006582	0,009532
200	0,008000	0,006930	0,009189	0,006618	0,009576

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