Particle Analysis Report of Company Name Simulator Fluids (A, B, C, D, E, F) from 1 to 10 million cycles

Protocol Summary: Particle size in the following documentation refers to diameter of an equivalent sphere. Analysis of the wear testing fluids for size characterization was performed after every million cycles up to 10 million cycles. The resultant samples were examined using laser diffraction (Low Angle Laser Light Scattering, LALLS) for quantitative number and volume particle size distributions and scanning electron microscopy (SEM) for qualitative analysis of characteristic particle shapes, following concentration and ultrasonic dispersion, as well as scanning electron microscopy (SEM) using energy-dispersive x-ray analysis (EDAX) microprobe analysis. All fluids used in processing were 0.2μ filtered and all processing was conducted in a Class 100 sterile hood. All particle characterization analyses met or exceeded the standards stipulated in ASTM F1877-05, Standard Practice for Characterization of Particles. SEM photographs of representative particles were also taken for each test specimen at each test interval.

LALLS Size Summary: The particle analysis for all six specimens demonstrated a relatively large mean particle diameter. Compiling the results for all 6 specimens and all time points, the mean particle diameter (based on particle volume) was 48 microns (range 6-263 microns on a volume basis). When re-analyzed in the same manner but based on particle number rather than volume, the mean particle diameter was 3 microns (range 0.14-20, on a number basis) –see Table 1.

Particle Size Changes 0-10 million cycles: The size of the average particle did not significantly change when analyzed at each million cycles (0 to 10 million cycles), see

Appendix 1- Figure 2. The size of the generated particles remained relatively constant at 48 microns mv and 3 microns mn. There was a slight non-significant increase in the size of the particles over the course of testing (see Figure 3a and 3b). This would tend to indicate that the mechanism of wear debris generation remains relatively constant over the course of 10 million cyclic loads. This is in contrast to articulating bearing surfaces, which typically change over time ("run-in") as does the micro-geometry of the counter faces. The average distribution of particle sizes for all 6 samples over the course of 10 million cycles is shown below for both the volume and number based analysis (Figure A and B) and is similar to those shown in Appendix 2.

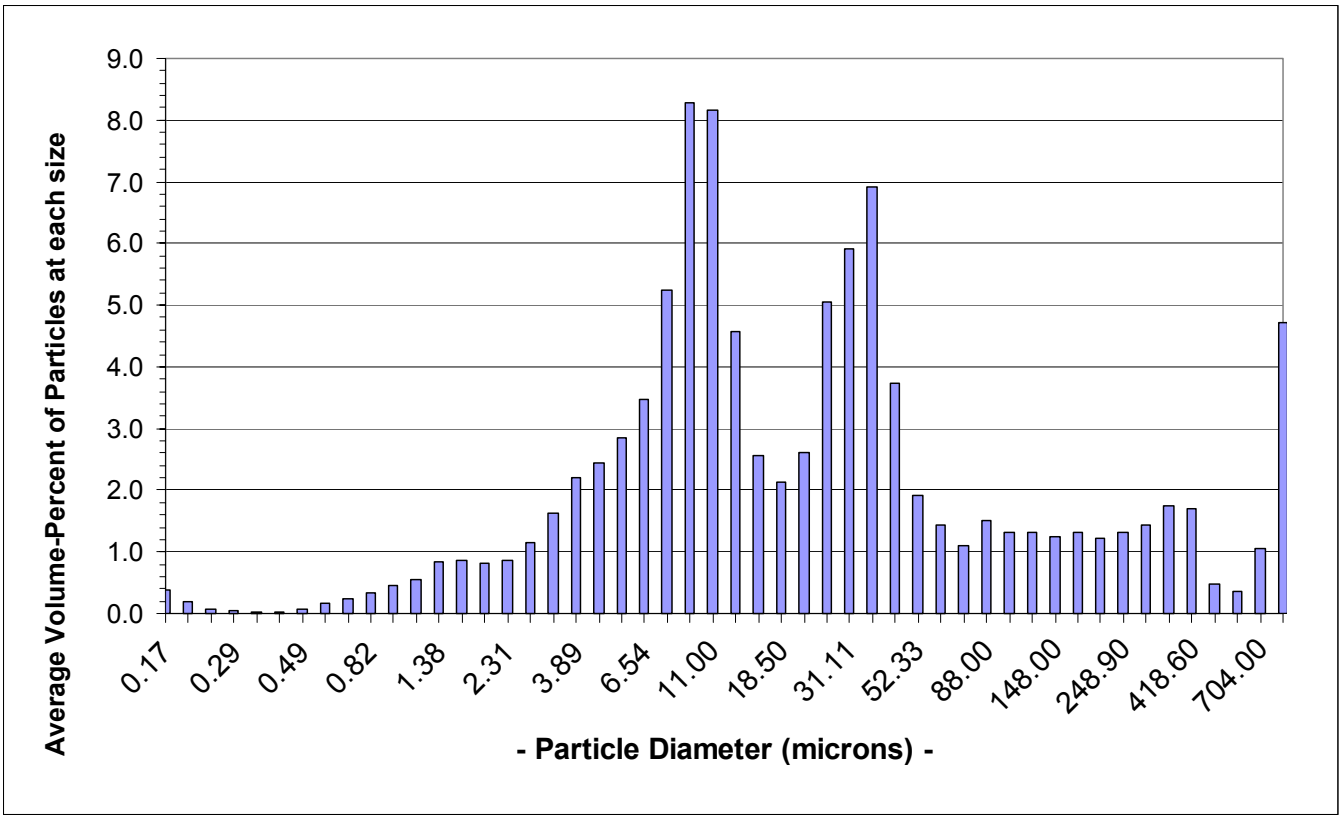


Figure A. The average volume-based percent distribution of particle sizes for 6 samples over 10 million cycles.

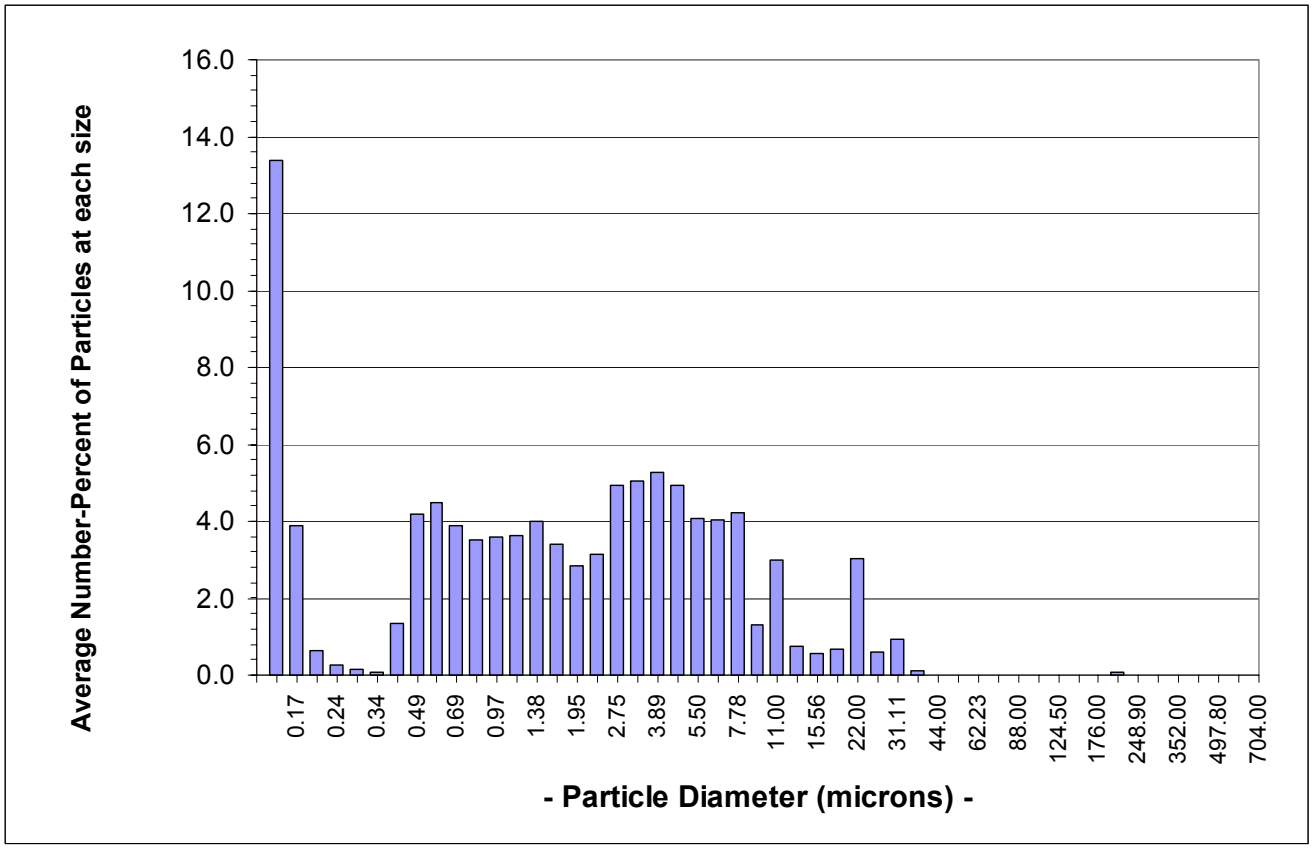


Figure B. The average number-based percent distribution of particle sizes for 6 samples over 10 million cycles.

Submicron Particle Distributions: Analysis of the percentage of submicron particles at each test interval was also performed. This analysis shows that the proportion of particles in the below 1,2 and 5 microns range were relatively constant over the course of the 10 million cycle testing at about 3%, 7% and 19% respectively. This is illustrated in Figure 4a, 4b and 4c and in the histograms included in the attachment to the test report in **Appendix Test Report**.

Total Numbers of Particles: The average total number of particles generated over the course of ten million cycles by a single sample was approximately $0.43x10^9$ particles/mg. The average number distribution of particles per mg of wear debris is shown in Figure C.

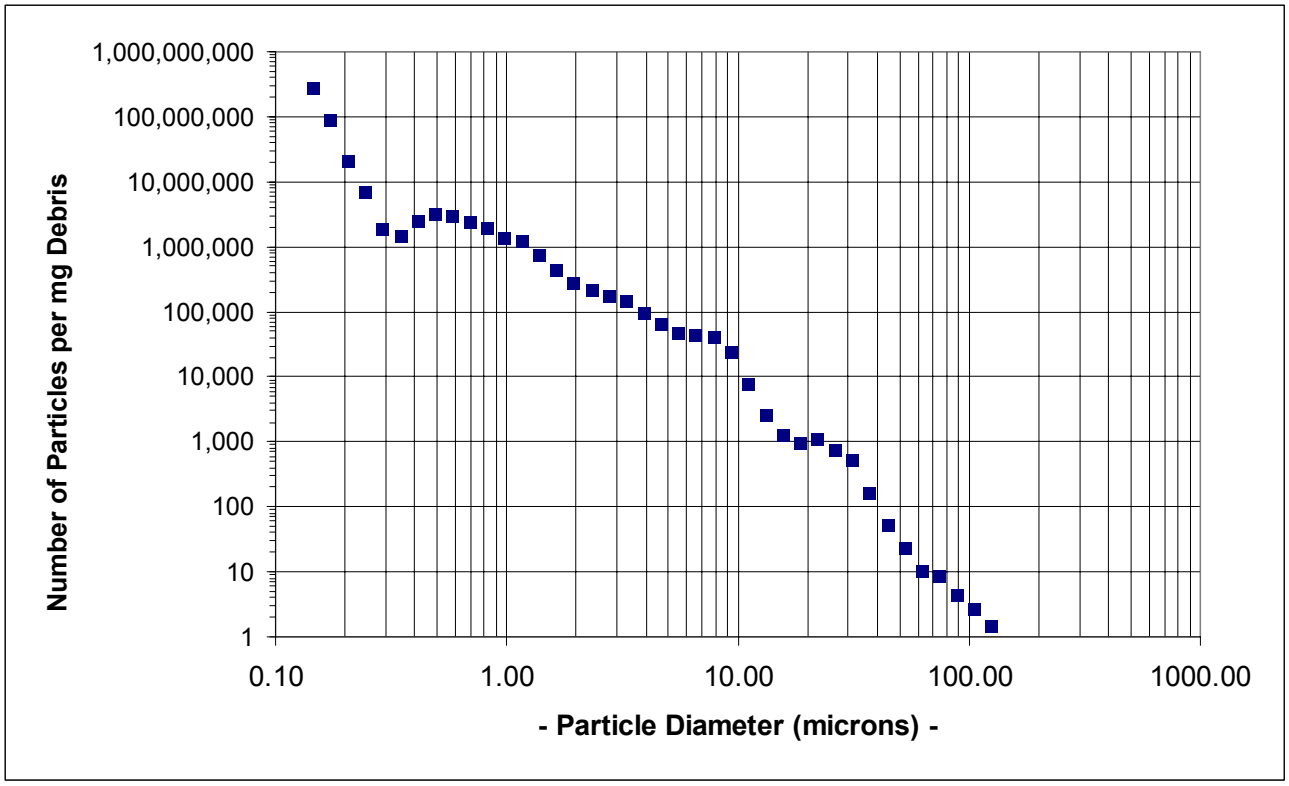


Figure C. A distribution showing an average of the total number of particles for each particle size per mg of wear.

SEM/EDAX Analysis: SEM analysis of the particles revealed that the vast majority of particles above 5 microns were flakes while the smaller particles below 5 microns were generally granular. EDAX analyses of particles demonstrated that the vast majority of the particles were Steel, Iron-oxide, Cobalt-alloy, and Titanium-alloy in composition. The vast majority of the particles identified on EDAX were Steel or iron-oxide particles, much fewer in number were particles of Co-alloy. The ratio of Co-alloy to Fe based particles was less than 1 to 10. The ratio of particles that were steel or iron-oxide was approximately 1 to 1. Ti-alloy flakes were very rare (less than 1 to 50: Ti-alloy to steel).

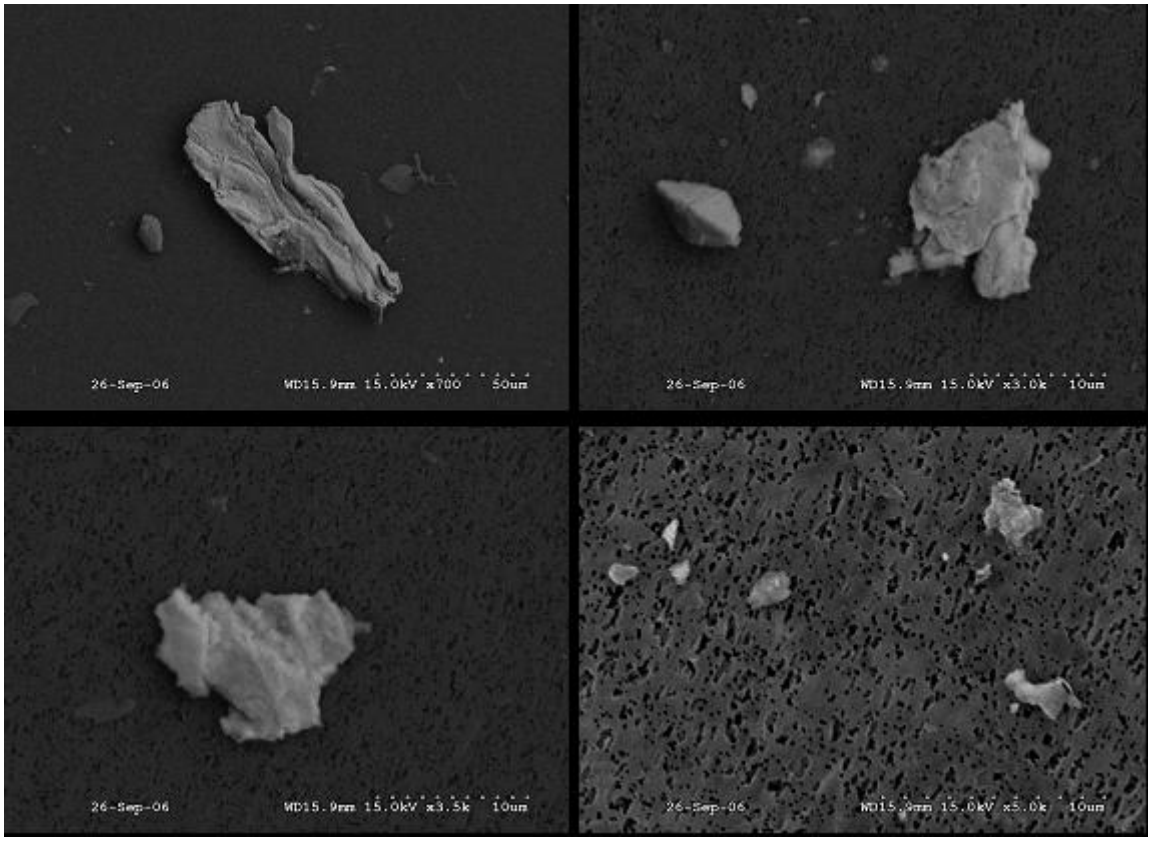


Figure E. Examples of metal particles from sample D (4 million cycles) showing flake and granular particle morphology.

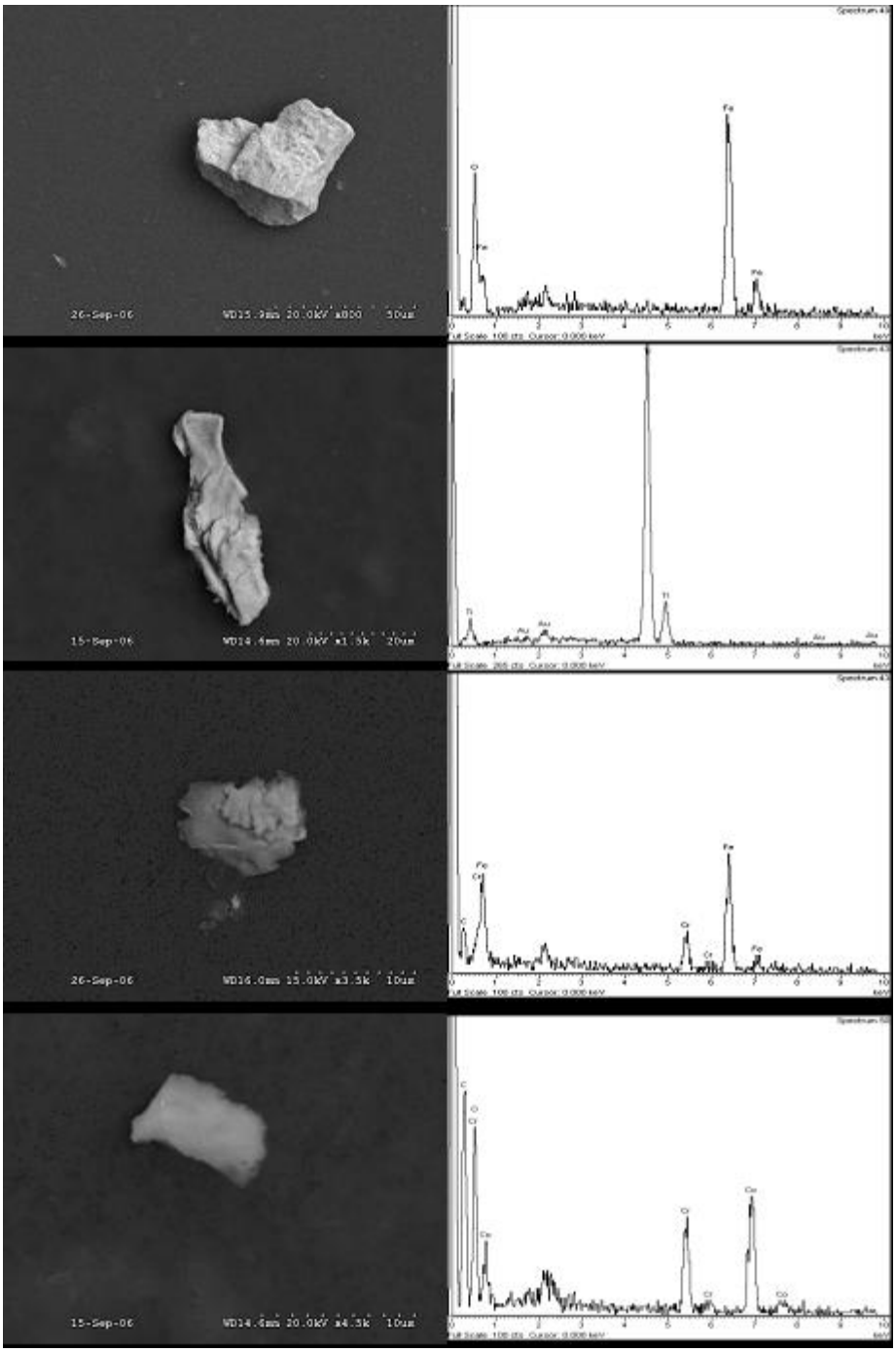


Figure F. Examples of typical particles EDAX identified as Fe-oxide, Ti-alloy, steel and Co-alloy (Top to Bottom) from sample simulator fluids (Note Au and PD were used for sputter coating specimens for SEM). Generally the Co-alloy particles tended to be smaller and more granular in shape (<10um), but were not heavily oxidized, i.e. not likely corrosion products.

SEM vs. LALLS: Particle size analysis of retrieved implant debris (ASTM 1877) has typically been conducted by particle counting using scanning electron microscopy (SEM). Technologies such as Laser Diffraction (LD), which provide both volume and number distributions, are the technical standard for size analysis of pharmaceutical powders. SEM particle counting and Low Angle Laser Light Scattering (LALLS or laser diffraction) analysis are not equivalent in that volume analysis can provide number basis analysis but the reverse is not true (number analysis cannot be used to produce a volume based distribution of particle sizes). Number based analysis methods generally only identify the most numerous particles and thus are biased towards smaller particles identified in high magnification images because their average is typically weighted at over 10,000 times that of the low magnification SEM images for distribution calculations. SEM analysis (like a coulter analysis) does provide for an indirect calculation of total debris per volume and provide a measure of shape (e.g. aspect ratio) where typically LALLS does not. Laser diffraction analysis measures millions to billions of particles, yet lacks the capability to yield morphologic data, e.g. aspect ratios of specific size subsets within any given sample. Thus it depends on what critical distribution characteristics are desired that determines which is/are the best methods of particle characterization. Generally, the greater the percent mass of small particles (i.e. <5um), the greater is the agreement between the two techniques.

Conclusions: The serum/saline simulator fluid samples were extremely clear of debris, bacterial/fungus contamination and protein precipitation upon arrival. The debris

associated with post-enzyme processing was close to the detection limit of the laser diffraction analyzer (Microtrac x-100), which is approx 0.1mg metal debris. The average particle size of all six samples over the course of 0 to 10 million cycles remained relatively constant at approximately 48 microns (mv, volume analysis) and 3 microns (mn, number analysis) in diameter. The percentage of <5 micron and submicron particles were relatively constant and only slightly decreased over the 10 million cycles of testing.

Appendix 1

Particle Size Characteristics

Compiled results of these analyses are discussed in the following.

1. The average particle size generated over the course of testing using the combined results of all specimens was…

Average Diameter (volume)	Average Diameter (number)	Average Volume Percent of Small (phagocytosable) Particles		
Mv	Mn	<1um	<2um	<5um
48.44um	3.20um	2.68%	6.52%	17.57%

   a. **48.44 microns** (mv, volume analysis) and **3.20 microns** (mn, number analysis)
   b. **3%** of particles were submicron in size, **7%** were below 2 microns, and **18%** were below 5 microns in size.

   Volume analysis is biased by the percentage of total cumulative volume (or mass), a particle represents and *Number analysis* is biased by the percentage of total number, a particle represents. Both analyses were conducted to ensure comprehensive particle distribution. Generally volume analysis more accurately depicts the majority of lost volume from a given implant.
   Note: Volume analyses values (mv) were disregarded at Near Detection Limit (NDL) measurements (equivalent to <0.15mg).

2. Average particle sizes for all specimens and all cycles ranged from **6.76 to 263.4 microns (volume basis)** and **0.14 to 20.46 microns (number basis)** (Table 1).
   Note: Volume analyses values (mv) were disregarded at Near Detection Limit (NDL) measurements

	A		**B**		**C**		**D**		**E**		**F**	
	Mean diameter (volume)	Mean diameter (number)	Mean diameter (volume)	Mean diameter (number)	Mean diameter (volume)	Mean diameter (number)	Mean diameter (volume)	Mean diameter (number)	Mean diameter (volume)	Mean diameter (number)	Mean diameter (volume)	Mean diameter (number)
Million Cy	**mv**	**mn**	**mv**	**mn**	**mv**	**mn**	**mv**	**mn**	**mv**	**mn**	**mv**	**mn**
1	7.308	0.7	8.149	0.719	6.921	0.7	7.007	0.813	6.763	0.854	6.833	0.739
2	12.29	0.785	30.8	0.877	13.25	1.306	*NDL*	4.015	32.51	5.832	16.62	4.492
3	25.1	8.551	82.93	20.46	249.7	9.7	16.94	6.767	18.85	15.52	149.8	4.956
4	23.41	0.15	13.83	6.622	*NDL*	6.783	*NDL*	6.522	13.28	6.7476	30.33	7.53
5	32.39	0.147	15.24	0.139	30.13	2.579	7.426	3.003	27.67	0.139	9.943	0.146
6	22.64	0.141	*NDL*	0.138	10.19	0.142	18.08	4.813	230.5	1.968	*NDL*	4.153
7	40.63	4.122	*NDL*	4.447	*NDL*	6.273	*NDL*	4.933	33.09	0.975	13.09	0.845
8	31.92	0.627	19.19	0.139	20.54	2.075	34.86	2.365	182.4	1.202	19.77	0.613
9	85.87	0.529	103.4	0.726	18.71	0.837	263.4	0.185	*NDL*	2.093	*NDL*	2.103
10	*NDL*	0.718	*NDL*	1.878	*NDL*	3.609	*NDL*	3.782	31.64	0.786	*NDL*	4.149

Table 1. Average particle size for each of the six specimens at each range of testing.

mv – Mean Diameter, in microns, of the **Volume** distribution – represents the center of gravity of the distribution. Implementation of the equation used to calculate mv will show it to be weighted (strongly influenced) by coarse particles. It is a type of "average particle size."
mn – Mean Diameter, in microns, of the **Number** distribution – is calculated using the volume distribution data and is weighted to the small particles. This type of "average particle size" is related to population

3. The range of particle sizes (identified peaks in the size distributions, Figure 1), did not change over the course of 10 million cycles for all six samples. These peaks, that represent the different size particles within the samples, varied between 1 and 5 for all samples. To what extent other non-consistent distribution characteristics are indicative of contamination or load-cycle-specific behavior is unknown at this time.

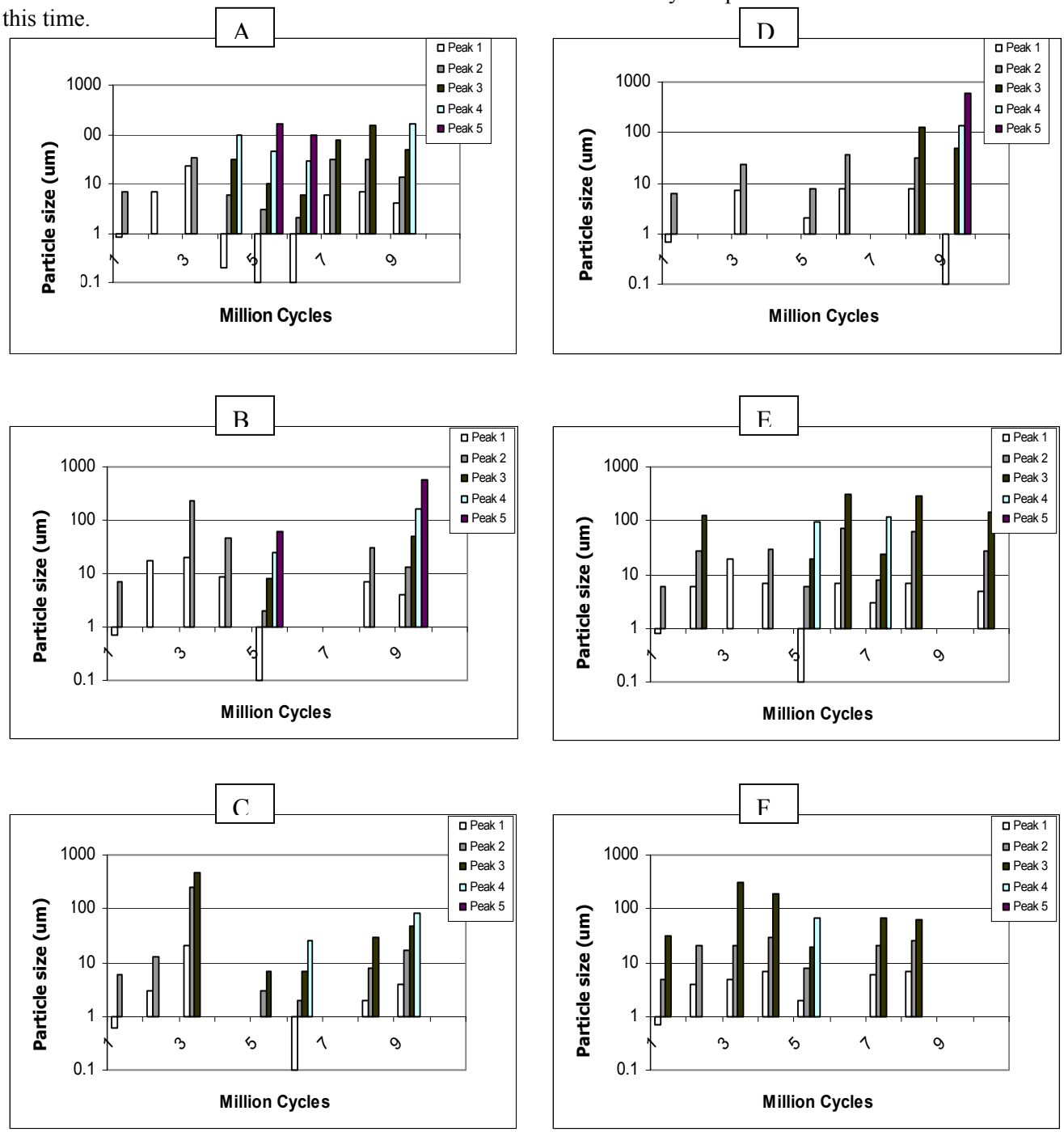


Figure 1. Graphical representation of the number of peaks within each particle size distribution and their associated particle size for each of the six specimens over the course of testing. Variation in the number of peaks over the course of testing shows the heterogeneity and complexity of wear debris production was consistent and that a reduction in wear to a single peak (particle size) did not occur in any of the specimens.

4. A graphical representation of the average particle size using both mv (volume) and mn (number) analysis for each of the six specimens over the course of testing can be seen in Figure 2. The average particle size of all six specimens (mv, mn) demonstrated relatively consistent size over the course of testing to a size of roughly 48 microns (mv, volume basis) and 3 microns (mn, number basis) in diameter (Figures 3a, 3b).

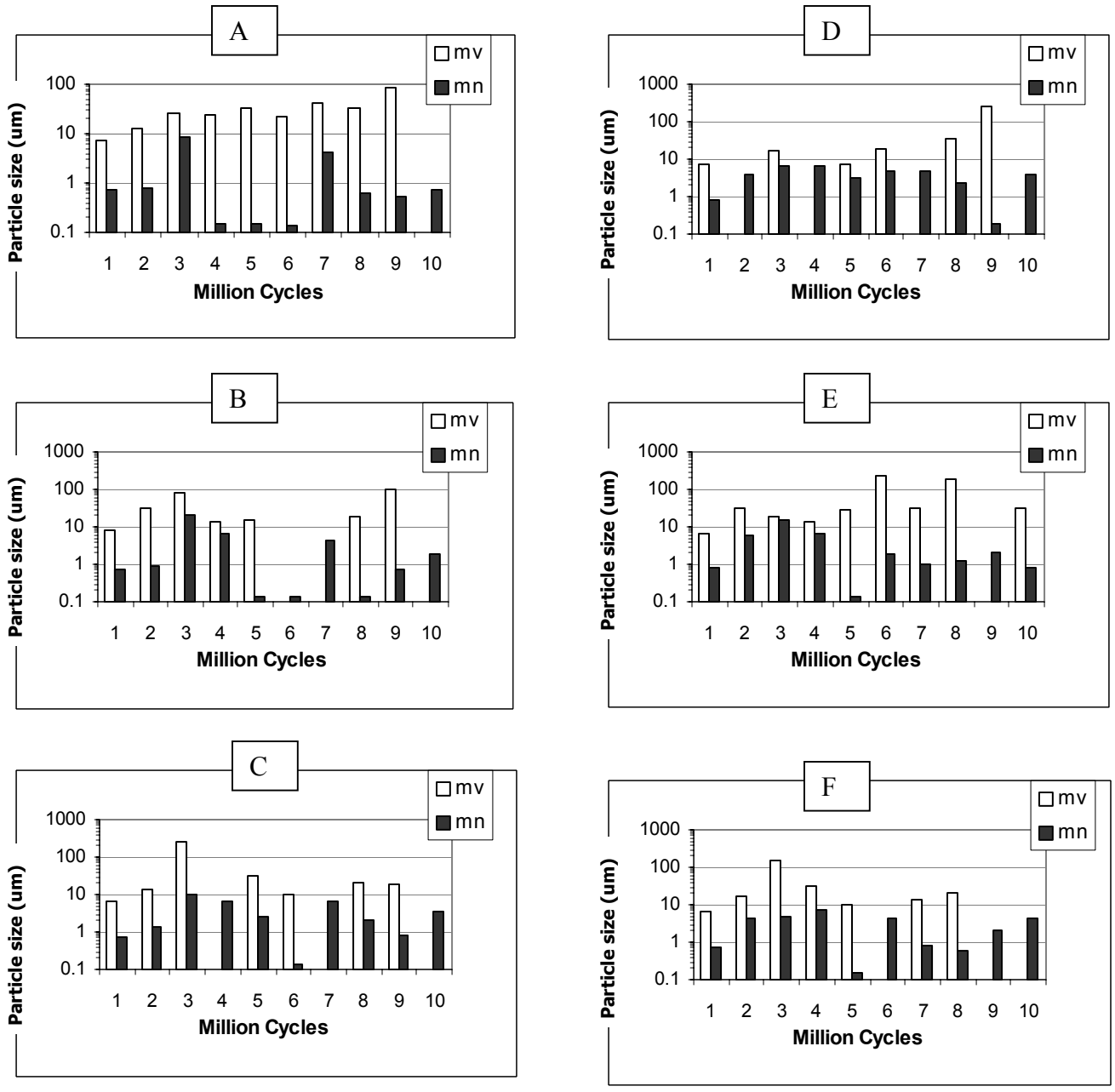


Figure 2. Graphical representation of the average particle size using both mv (volume) and mn (number) analysis, for each of the six specimens over the course of testing.

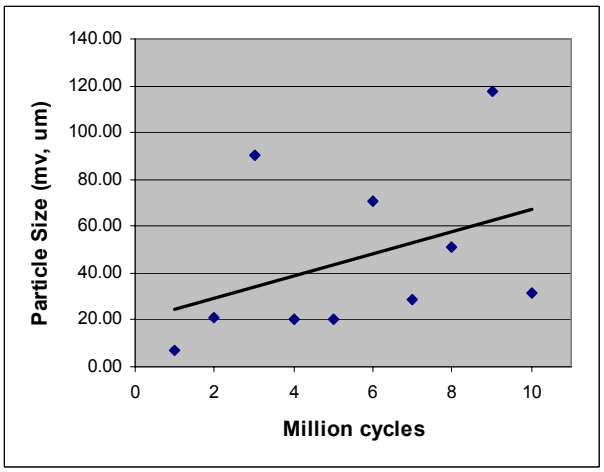


Figure 3a. Volume basis (mv)

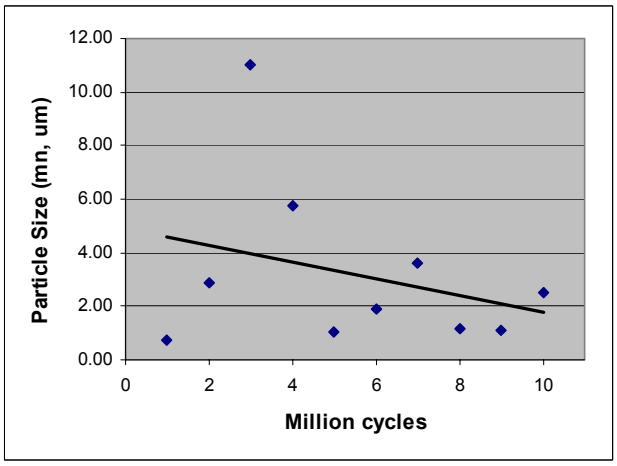


Figure 3b. Number basis (mn)

5. The average debris pattern of the six specimens demonstrated a general decrease in percentage of submicron particles over the course of testing, Figure 4a and Table 2. Similarly, the average percentage of particles below 2 and 5 microns generally decreased over the course of testing, Figures 4b and 4c.

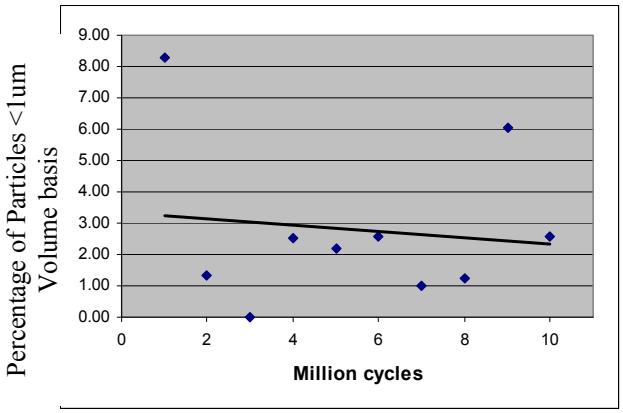


Figure 4a. Samples <1um.

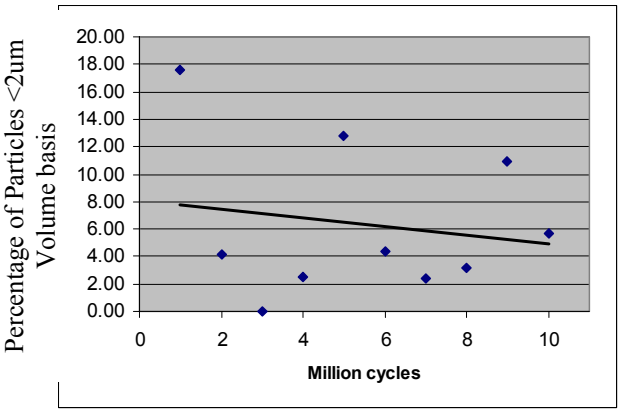


Figure 4b. Samples < 2um.

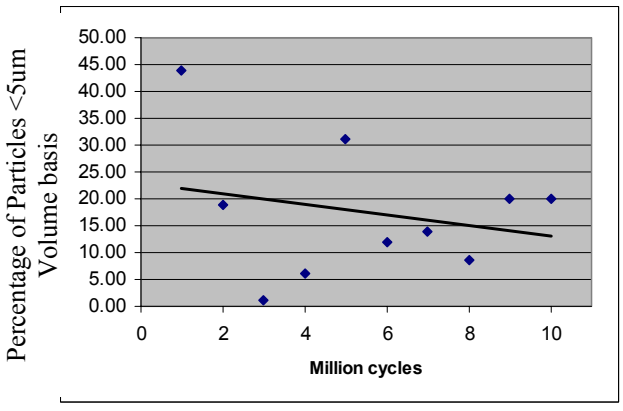


Figure 4c. Samples < 5um.

Table 2. Percentage of particles smaller than the denoted size (volume basis), for each of the six specimens over the course of testing. (NDL=Near Detection Limit of LALLS analysis)

	A		B		C	
	Mean diameter (volume)	Mean diameter (number)	Mean diameter (volume)	Mean diameter (number)	Mean diameter (volume)	Mean diameter (number)
Million Cycles	**mv**	**mn**	**mv**	**mn**	**mv**	**mn**
1	7.308	0.7	8.149	0.719	6.921	0.7
2	12.29	0.785	30.8	0.877	13.25	1.306
3	25.1	8.551	82.93	20.46	249.7	9.7
4	23.41	0.15	13.83	6.622	*NDL*	6.783
5	32.39	0.147	15.24	0.139	30.13	2.579
6	22.64	0.141	*NDL*	0.138	10.19	0.142
7	40.63	4.122	*NDL*	4.447	*NDL*	6.273
8	31.92	0.627	19.19	0.139	20.54	2.075
9	85.87	0.529	103.4	0.726	18.71	0.837
10	*NDL*	0.718	*NDL*	1.878	*NDL*	3.609

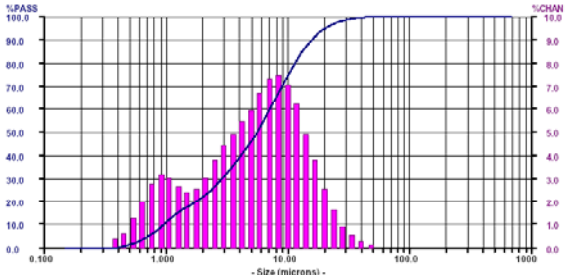
	D		E		F	
	Mean diameter (volume)	Mean diameter (number)	Mean diameter (volume)	Mean diameter (number)	Mean diameter (volume)	Mean diameter (number)
Million Cycles	**mv**	**mn**	**mv**	**mn**	**mv**	**mn**
1	7.007	0.813	6.763	0.854	6.833	0.739
2	*NDL*	4.015	32.51	5.832	16.62	4.492
3	16.94	6.767	18.85	15.52	149.8	4.956
4	*NDL*	6.522	13.28	6.7476	30.33	7.53
5	7.426	3.003	27.67	0.139	9.943	0.146
6	18.08	4.813	230.5	1.968	*NDL*	4.153
7	*NDL*	4.933	33.09	0.975	13.09	0.845
8	34.86	2.365	182.4	1.202	19.77	0.613
9	263.4	0.185	*NDL*	2.093	*NDL*	2.103
10	*NDL*	3.782	31.64	0.786	*NDL*	4.149

Appendix 2

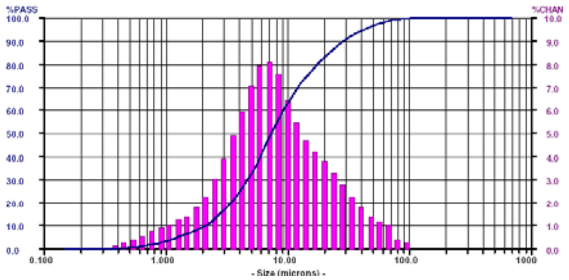
Volume Based Particle Distributions for A, B, C, D, E, F from 1 to 10 million cycles

A - Volume based size distributions

1M



2M



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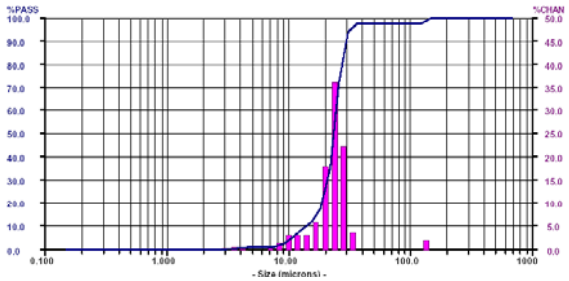
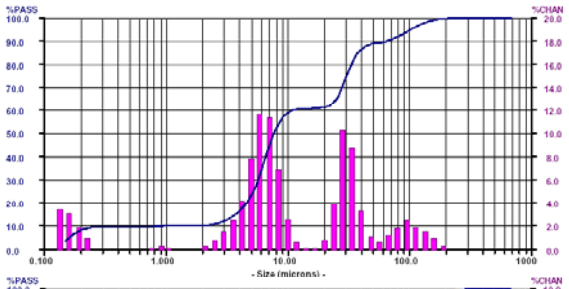
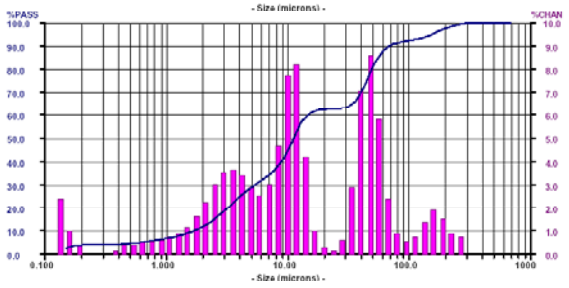


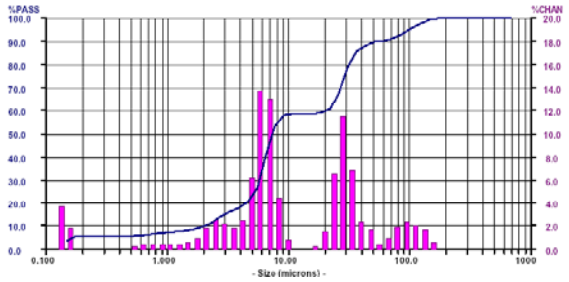
4M



5M



6M



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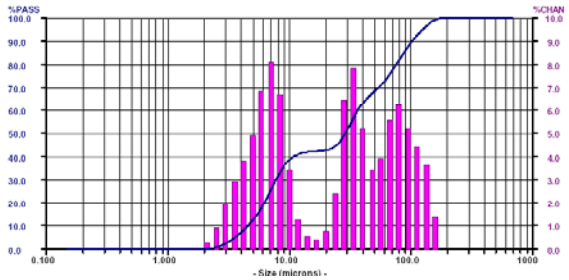

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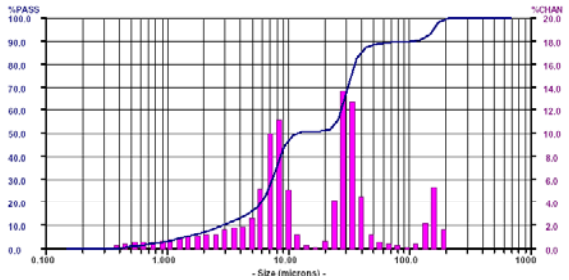

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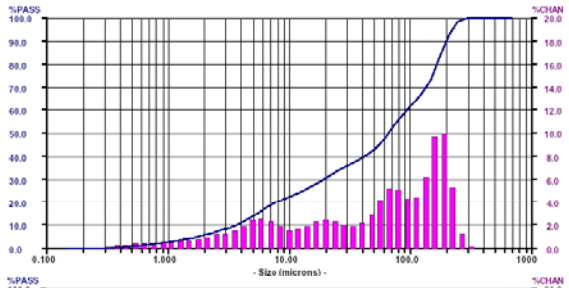
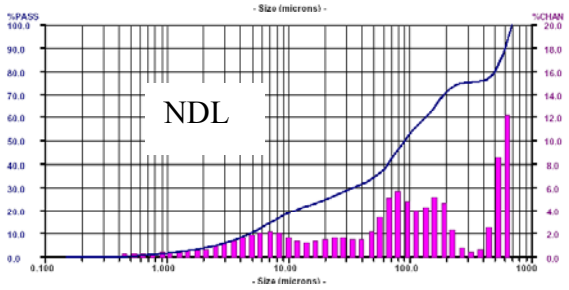
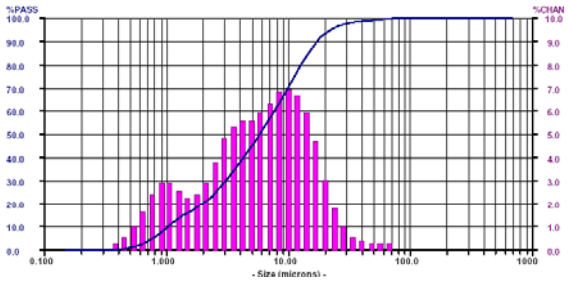


10M



B - Volume based size distributions

1M



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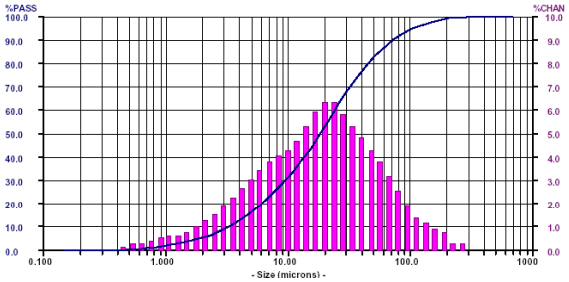

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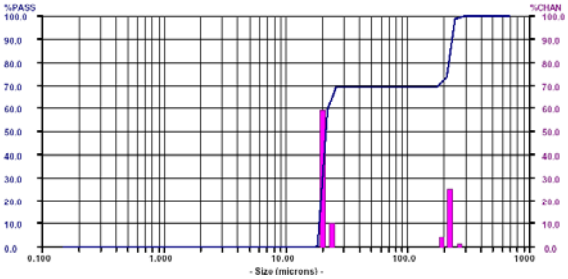

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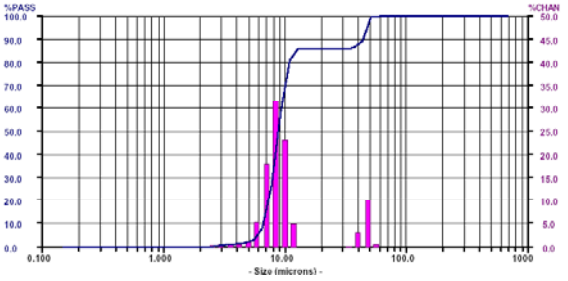

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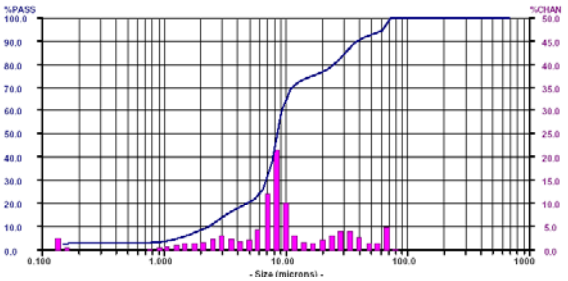

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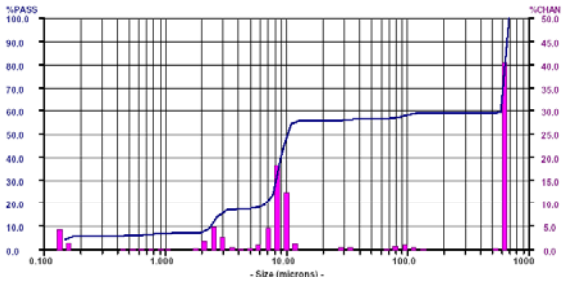

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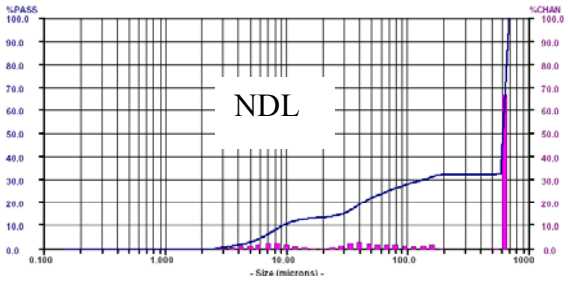

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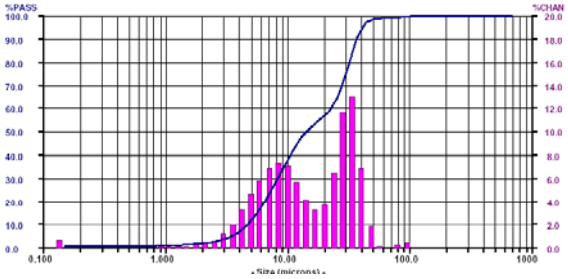

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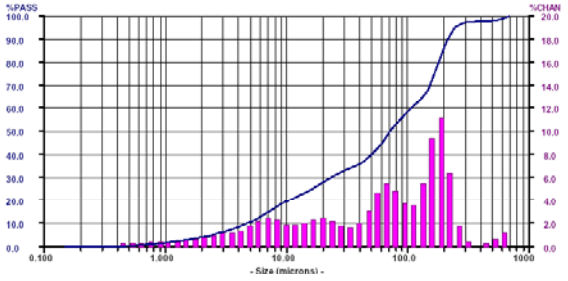
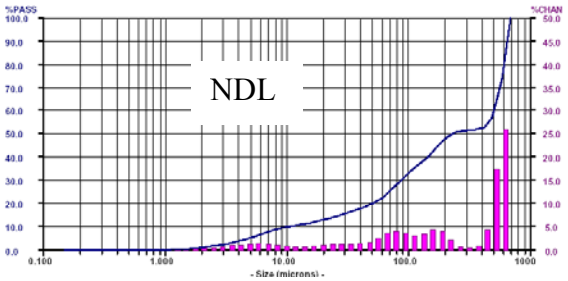


10M



C - Volume based size distributions

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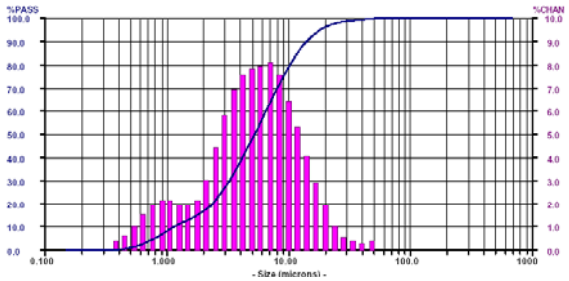

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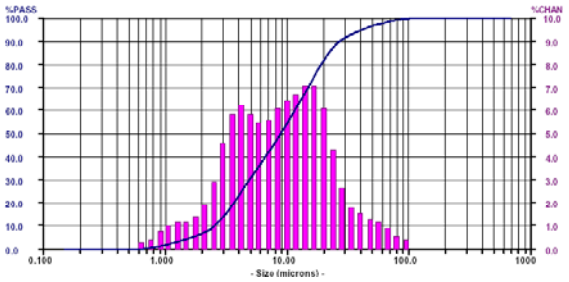

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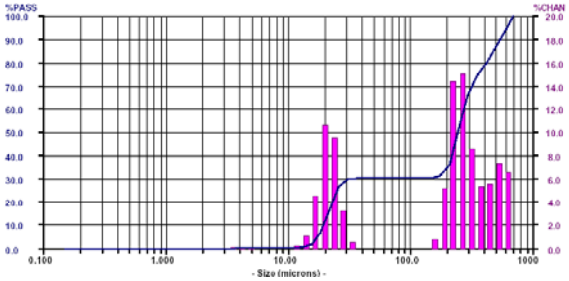

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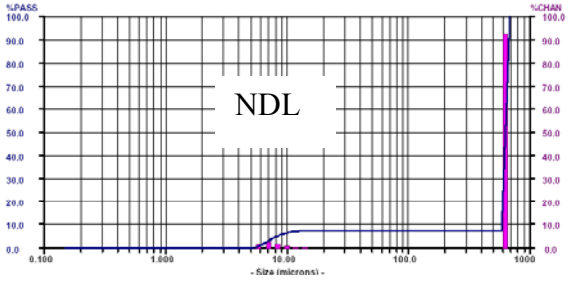

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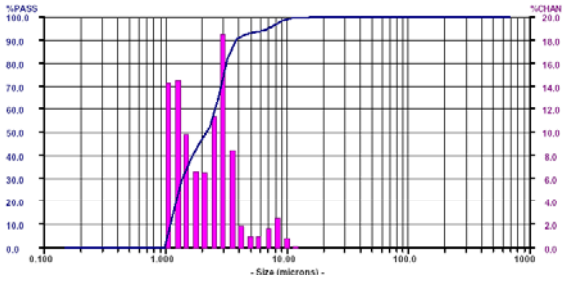

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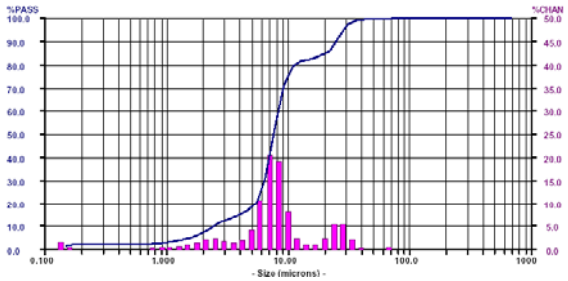

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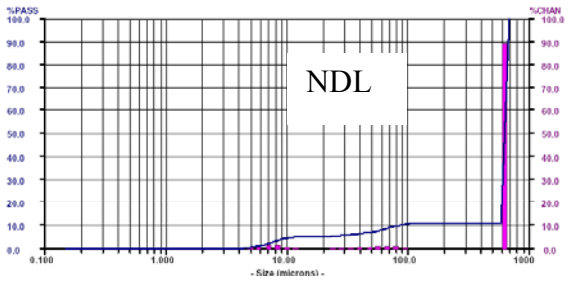

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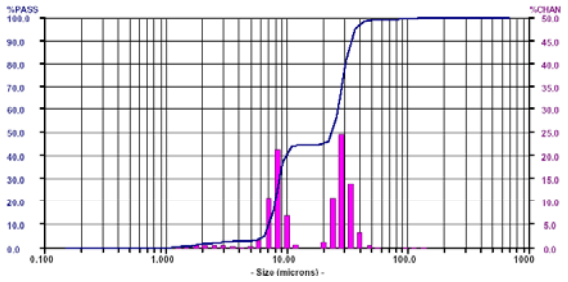

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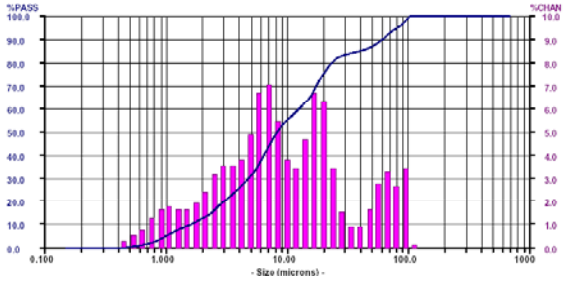

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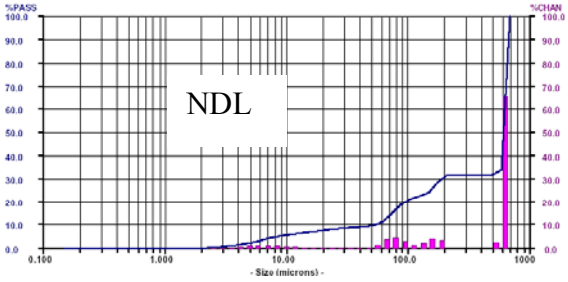

D - Volume based size distributions

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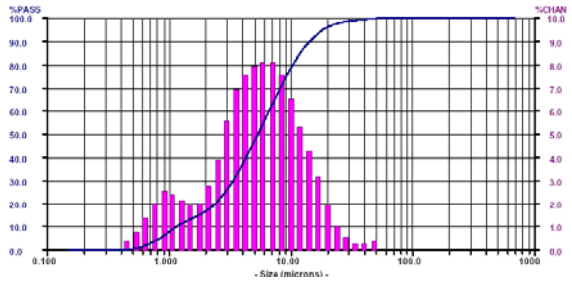

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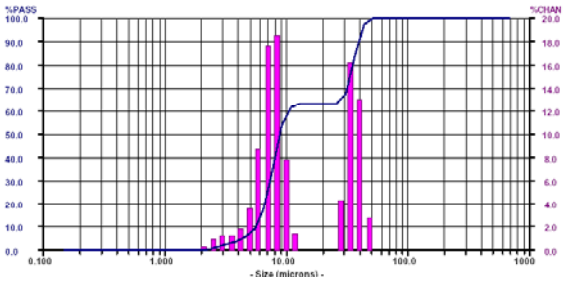

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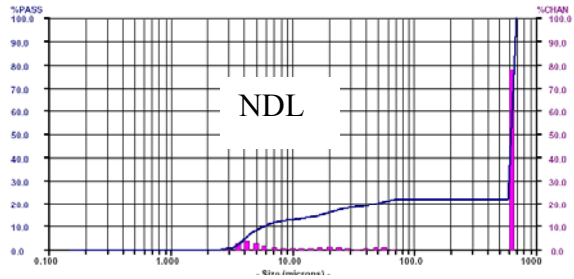

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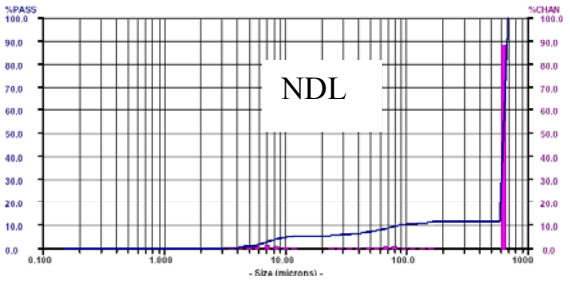

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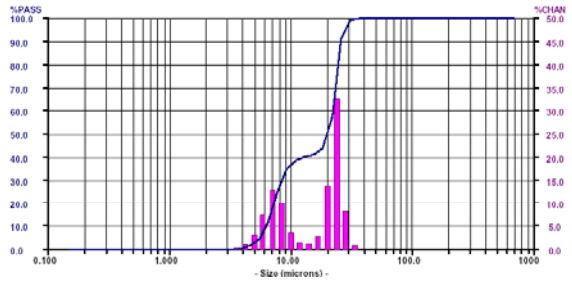

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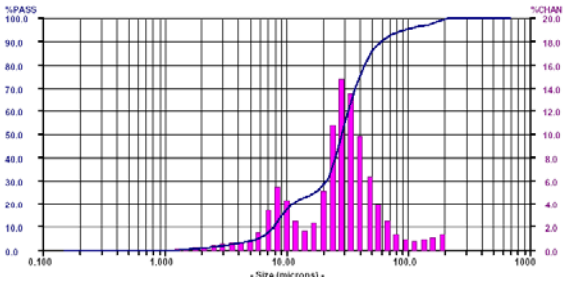

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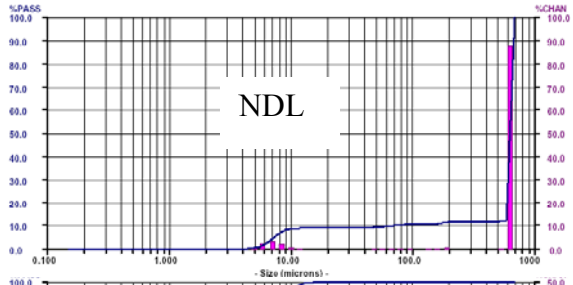

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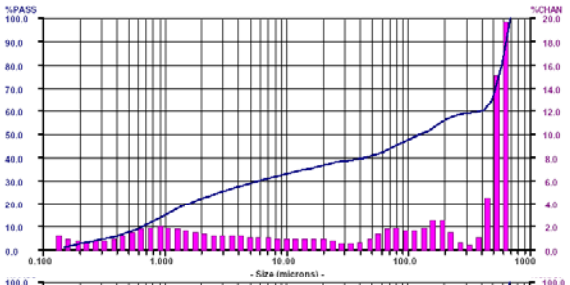

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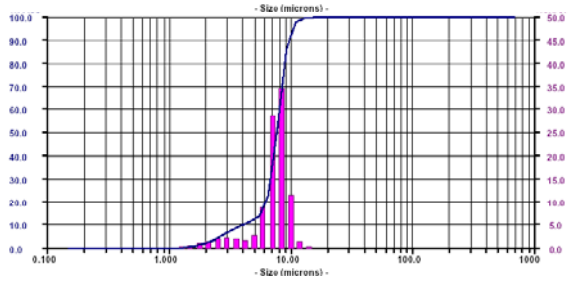

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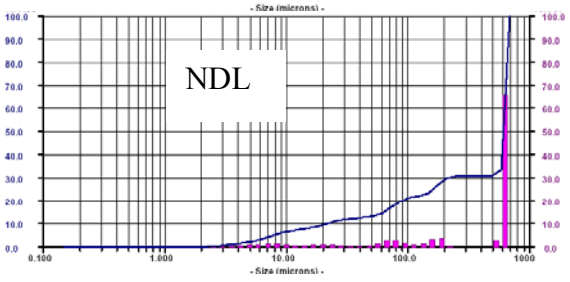

E - Volume based size distributions

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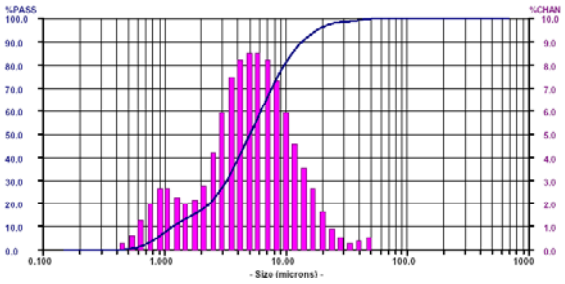

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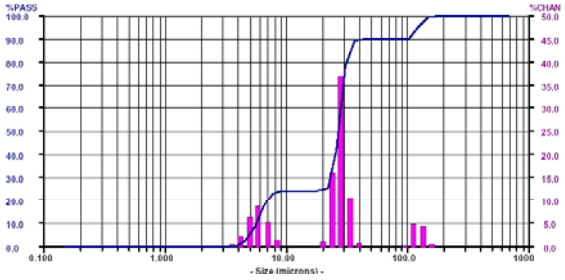

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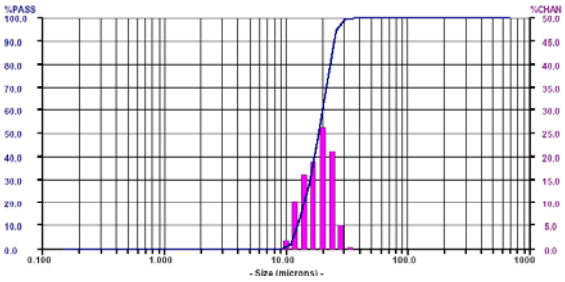

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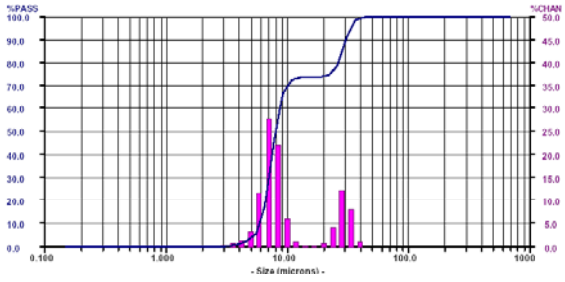

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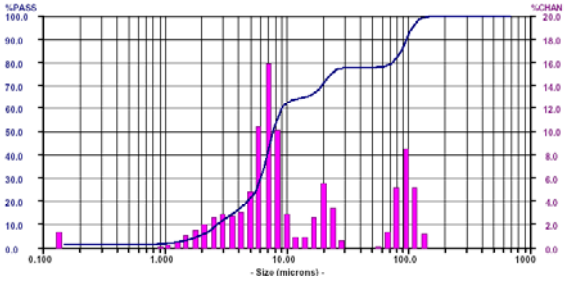

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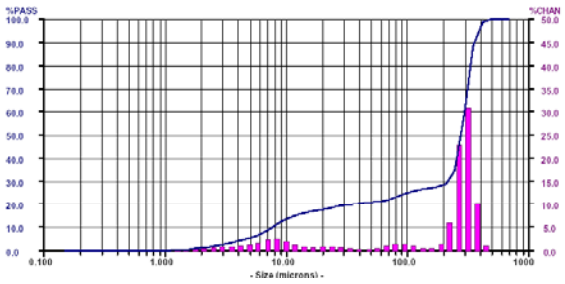

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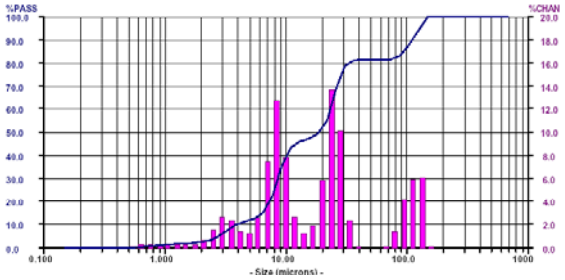

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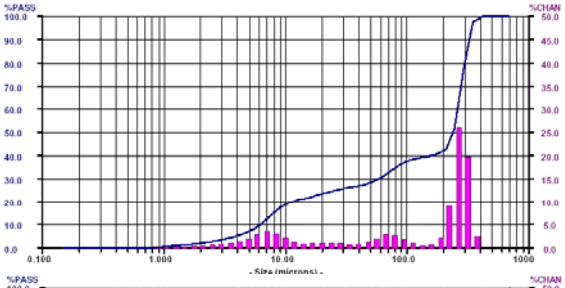

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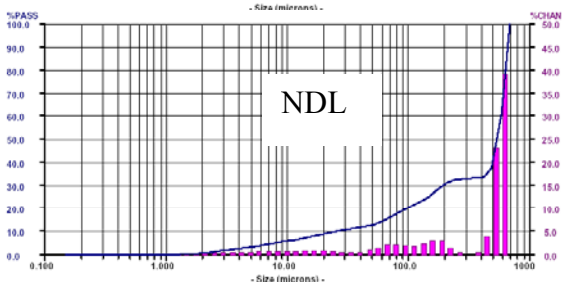

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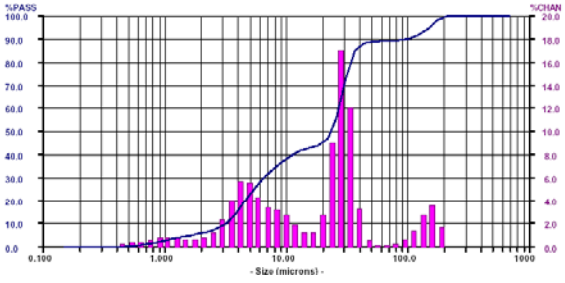

F - Volume based size distributions

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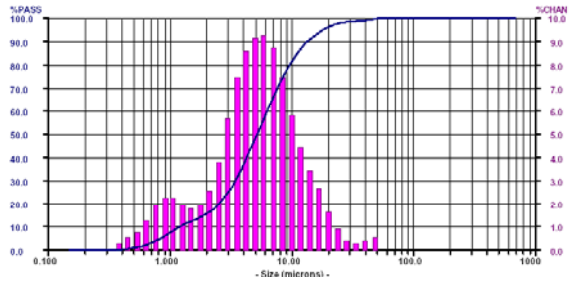

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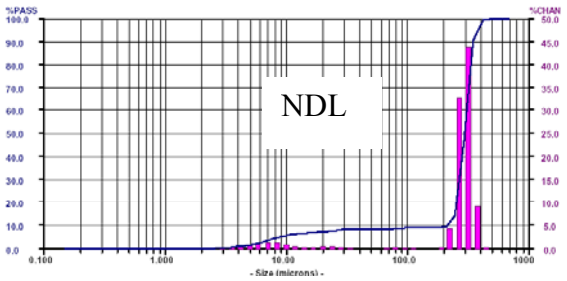

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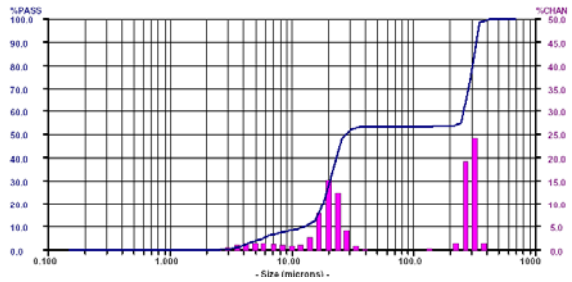

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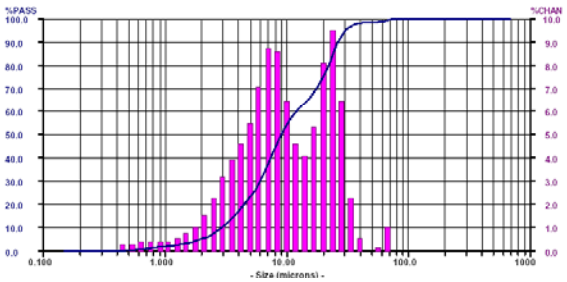

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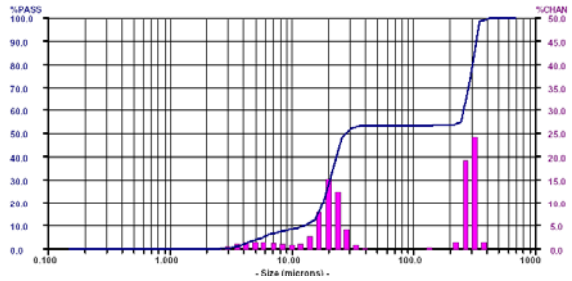

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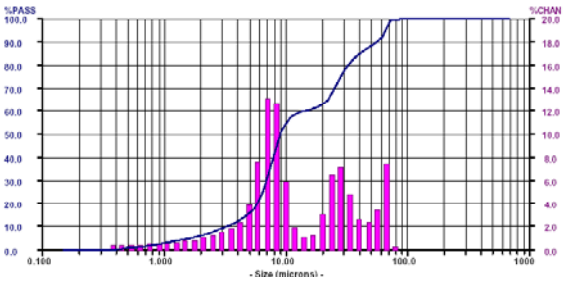

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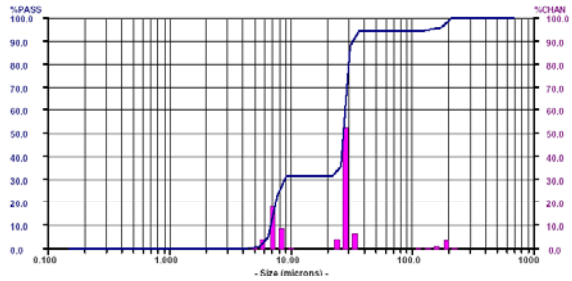

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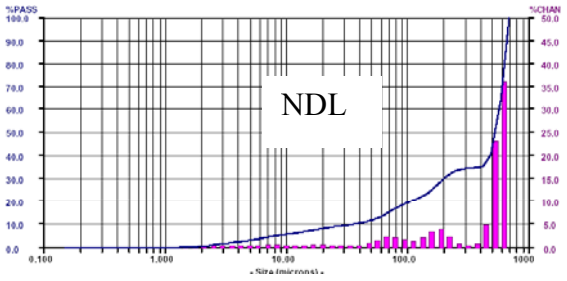

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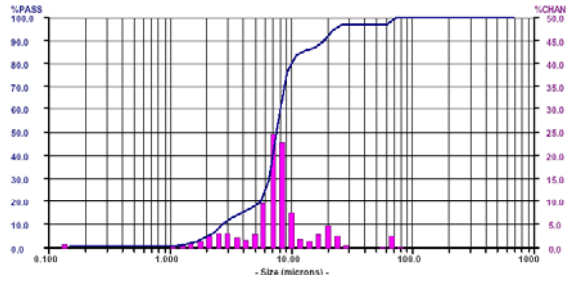

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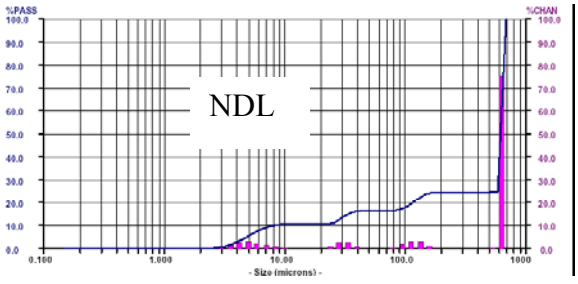

Appendix 3

DESCRIPTION: PARTICLE ANALYSIS SAMPLE PREPARATION AND TEST METHOD

1.0 SCOPE:

This standard briefly describes the method used for preparing proteinaceous samples for particle size analysis using the Microtrac X-100 analyzer and SEM/EDAX analysis.

2.0 PRINCIPLE:

Standardized, easily repeatable, user independent, proper sample preparation is critical in order to report accurate and consistent results.

The sample must be representative of the entire product lot or batch.

3.0 REFERENCES:

3.1 BASIC PRINCIPLES OF PARTICLE SIZE ANALYSIS written by Dr. Alan Rawle,Malvern Instruments Limited, Enigma Business Park, Grovewood Road, Malvern, Worcestershire, WR14 1XZ, UK. Tel: +44 (0)1684 892456 Fax: +44 (0)1684 892789

3.2 Michael D. Ries, Marcus L. Scott, and Shilesh Jani, Relationship Between Gravimetric Wear and Particle Generation in Hip Simulators: Conventional Compared with Cross-Linked Polyethylene, J. Bone Joint Surg. Am., Nov 2001; 83: 116 - 122.

3.3 Comparison of three joint simulator wear debris isolation techniques: Acid digestion, base digestion, and enzyme cleavage, S. Niedzwiecki, C. Klapperich, J. Short, S. Jani, M. Ries, L. Pruitt, J Biomed Mater Res. 2001 Aug;56(2):245-9.

3.4 Campbell P, Ma S, Yeom B, McKellop H, Schmalzried TP, Amstutz C. Isolation of predominantly submicron-sized UHMWPE wear particles from periprosthetic tissues J Biomed Mater Res 1995;29:127–131.

3.5 Landry ME, Blanchard CR, Mabrey JC, Wang X, Agrawal CM. Morphology of in vitro generated ultrahigh molecular weight polyethylene wear particles as a function of contact conditions and material parameters. J Biomed Mater Res 1999;48:61–69.

3.6 Schmalzreid TP, Campbell P, Schmitt A, Brown I, Amstutz H. Shapes and dimensional characteristics of polyethylene wear particles generated in vivo by total knee replacements compared to total hip replacements. J Biomed Mater Res 1997;36: 203–210.

3.7 Ramamurti BS, Estok DM, Jasty M, Harris WH. Analysis of the kinematics of different hip simulators used to study wear of candidate materials for the articulation of total hip arthroplasties. J Orthop Res 1998;16:365–369.

3.8 Shanbhag AS, Jacobs JJ, Glant TT. Composition and morphology of wear debris in failed uncemented total hip replacements. J Bone Joint Surg 1994;76A:1664–1675.

3.9 ASTM 1877-05 Standard Practice for Characterization of Particles

3.10 ASTM D4464-00 Standard Test Method for Particle Size Distribution of Catalytic Material by Laser Light Scattering

4.0 APPARATUS:

4.1 Light Scattering Particle Size Distribution Analyzer (MicroTrac- X-100).
4.2 Light Microscope and Hemacytometer
4.3 Incubator 37 Degrees Celsius
4.4 Ultra-sound bath
4.5 Rocker Platform
4.6 Hot Plate
4.7 Deionized 0.1 micron filtered water
4.3 Disposable pipets, sample preparation vials (50 mL).
4.4 Centrifuge
4.5 Polycarbonate 0.1 micron filters
4.6 Filtering Apparatus.
4.7 Vacuum Pump
4.8 Desiccator
4.9 Dispersant. De-ionized H2O: A liquid that the material to be analyzed is insoluble in.
4.10 Enzyme Digestion: 0.1 micron filtered Trypsin 10X, Proteinase K
4.11 Acid Digestion (70% HCl)
4.12 Surfactant/Protein denaturant. 2% solution of sodium dodecyl sulfate in water and 0.5% Triton X (T-100).

5.0 PROCEDURE FOR PARTICLE ANALYSIS)

5.0-A SUMMARY

1. *All fluids used in processing are filtered at 0.2micron and/or tested to particulate free*
2. *All sample processing is conducting in a class II sterile environment.*

Enzyme method:

1. **Original >200mL fluid sample divided into eight 50mL tubes and centrifuged at 3500 rpm for 30 min. 2.5mL sediment from each tube re-combined to total 20 mL/sample of sediment (particles and protein).**
2. **5x Trypsin enzyme digestion for 24 hours**
3. **5% SDS and Proteinase K (50-200 mg/L or ug/mL) digestion for 24 hours.**
4. **Boiling of samples in Proteinase K and Triton X for <30 min**
5. **Ultra-sonicated to de-flocculate particles (approx 10min at 37°C)**
6. **If sinuous protein precipitate visibly evident in samples begin at step 2 again.**
7. **Particles analyzed using laser diffraction (9 mL of processed sample taken and placed in 25mL of DH2O (not ethanol to prevent any re-precipitation of protein) in the LD machine for a total of (9ml+25ml) approx 34mL total in LD machine).**
8. **9. SEM preparation (remaining 1 mL)**
   a. **Remaining 1 mL diluted into 9 mL ethanol and 0.5% TritonX to reduce protein precipitate on filter during SEM analysis.**
   b. **100 – 200 uL sample diluted into used to filter onto 0.1 micron polycarbonate filters under vacuum.**

c. Sample sputter coated with Ag-Pd and characterized using a Hitachi S-3000 SEM/EDS. .

1. Approximately 40mL retained and re-frozen at -20C for any future analysis.