Technical Overview

Dynamic Binding Capacity of Oligonucleotides on PLRP-S Columns and Stationary Phases

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Abstract

PLRP-S is a polymeric polystyrene/divinylbenzene (PS/DVB) reversed-phase material that is ideally suited to oligonucleotide separation and purification, and is available in a wide range of pore sizes and particle sizes. This technical overview is intended to provide guidance for the correct choice of pore size depending on the size of oligonucleotide with which you are working.

Introduction

Dynamic binding capacity (DBC) is a way of determining the saturation point of a liquid chromatography column when a solution containing an appropriate molecule is passed through. To assess the DBC of different pore size PLRP-S stationary phases, a range of oligonucleotides, 25, 50, 75, and 100 bases, were prepared at a known concentration. These solutions were pumped through the different columns until saturation point was exceeded.

It is a useful indication of the ability of a stationary phase to bind a molecule, and is therefore helpful in determining the most suitable stationary phase for purification purposes.[1]

Experimental

Reagents and chemicals

All reagents were HPLC grade or higher.

Oligonucleotides were custom synthesized and purchased from Integrated DNA Technologies.

Instrumentation

Agilent 1260 Infinity II quaternary LC system comprising:

- Agilent 1260 Infinity II quaternary pump (G1311B)
- Agilent 1260 Infinity II high-performance autosampler (G1367E) with sample cooler
- Agilent 1260 Infinity II thermostatted column compartment (G1316C)
- Agilent 1290 Infinity II diode array detector (G4212B)

Sample preparation

Oligonucleotide samples were dissolved to a final concentration of 1.0 mg/mL in mobile phase A and placed on the instrument as Eluent C.

Mobile phase preparation

- A stock solution of 1 M triethylamine acetate (TEAA) was prepared by dissolving 60.0 g of glacial acetic acid in 900 mL of Milli-Q water. 101.2 g of triethylamine was added slowly to the stirred solution. The volume was made up to 1 L.
- Mobile phase A was prepared from 100 mL of 1 M TEAA stock solution and 900 mL of water.
- Mobile phase B was prepared from 100 mL of 1 M TEAA stock solution and 900 mL of acetonitrile.

HPLC conditions for binding capacity are outlined in Table 1, and the oligo cleanup gradient profile is outlined in Table 2.

Sequence

Oligo cleanup gradient (60 minutes) × 2

Oligo binding (100% C until breakthrough)

Oligo cleanup gradient (60 minutes) × 4

Method conditions

Table 1. HPLC conditions for binding capacity.

Column	Agilent PLRP-S, 2.1 × 50 mm, 5 µm, 100, 300, 1,000, and 4,000 Å
Mobile Phase	Eluent A: 0.1 M TEAA Eluent B: 0.1 M TEAA in 90% ACN Eluent C: oligonucleotide solution
Flow Rate	0.21 mL/min
Column Temperature	25 °C
Detector	UV, 262 nm
Injection Volume	NA
Total Run Time	NA

Table 2. Oligo cleanup gradient profile.

Time	%A	%B
0.00	90.0	10.0
4.00	90.0	10.0
34.00	75.0	25.0
42.00	0.0	100.0
48.00	0.0	100.0
52.00	100.0	0.0
60.00	100.0	0.0

Results and discussion

PLRP-S stationary phases are fully porous polystyrene/divinylbenzene particles, which are inherently hydrophobic and suitable for reversed-phase. Available in a range of pore sizes (100, 300, 1,000, and 4,000 Å), where accessibility of very large molecules may be hindered in smaller pore sizes commonly seen across the market. Even though smaller pore-size particles have higher internal surface area, if the molecule is too large to fit into the pores, the capacity will be compromised.

To investigate more fully, four different oligonucleotides were custom synthesized (Table 3). This range of different sizes is representative of many oligonucleotide classes, from small microRNA to larger guide RNA. Deoxyribonucleotides were chosen to ensure higher stability for dynamic binding studies.

Initially, a barrel connector was used in place of a column to determine the delay volume. The instrument was flushed with 100% eluent A and then switched to 100% eluent C. The time taken before the oligonucleotide solution was detected at 262 nm was recorded as the dead volume of the system and then subtracted from each column run (Figures 1A to 1D). The measurement was taken at 25% full-scale deflection.

Table 3. Oligonucleotides used in this investigation.

25 mer	CATATAAGTTGCGTTACTTCGGCCT
50 mer	CCTAACCGCACCCTTAGCACGAAGA CAGATTCGTTCTTACCCATACTCCA
75 mer	CCGTTGGCAGGGGGATCGCATGTCC CACGTGAAACATTGCTAAACCCTCA GGTCTCTGAGCGACAAAAGCTTTAA
100 mer	AGGGAAATTCGCGCCCATAACTTGGT CCGAATACGGGTTCTTGCATCGTTC GACTGAGTTTGTTTTATATAAAACG GGCGCAATGTCTGCTTTGATCAAC

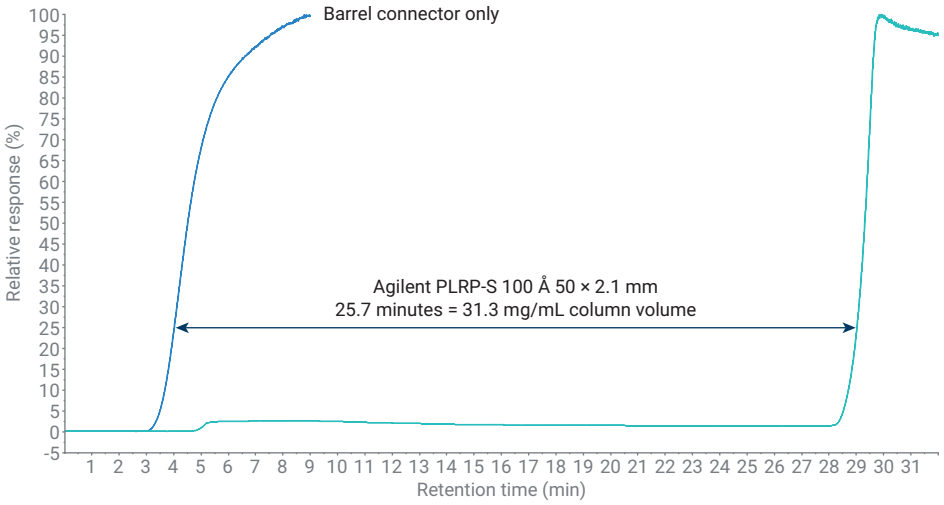


Figure 1A. 50 mer Oligonucleotide breakthrough with an Agilent PLRP-S 100 Å column.

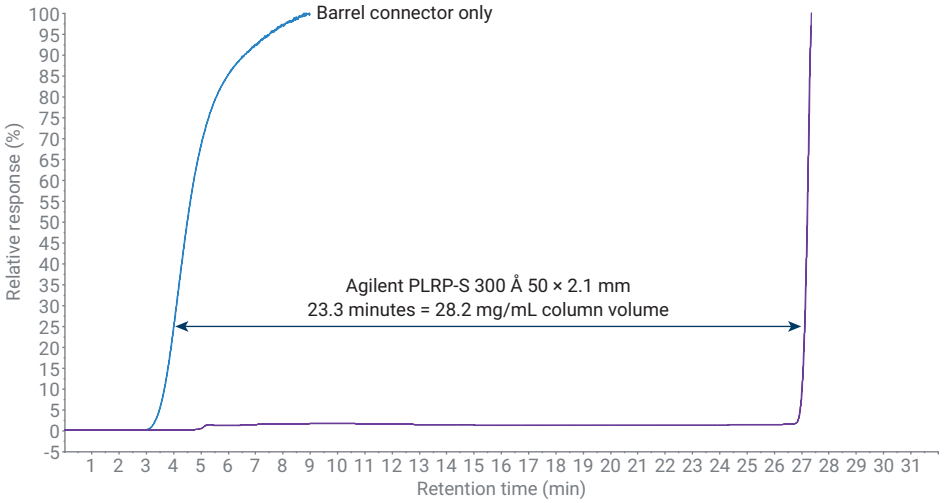


Figure 1B. 50 mer Oligonucleotide breakthrough with an Agilent PLRP-S 300 Å column.

Before performing the DBC, each column was subjected to a cleanup gradient lasting 60 minutes. Dynamic binding was measured when the oligonucleotide solution was detected to have saturated the column. Each column was then cleaned extensively by repeating the cleanup gradient four times, to remove any remaining bound oligonucleotide. It is important to recognize the importance of the cleanup gradient, and to ensure that the gradient is not too steep so that the oligonucleotide remains absorbed on the column. The gradient profile can be shown in Figure 2.

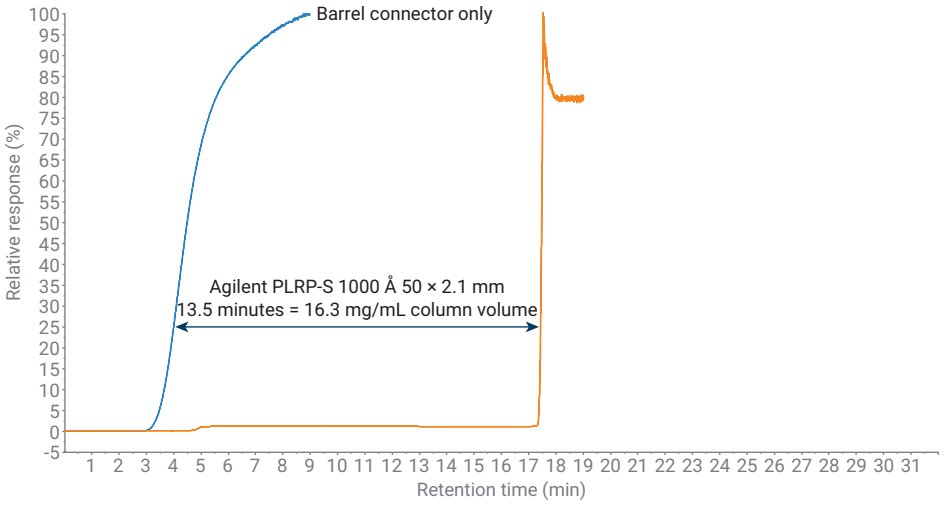


Figure 1C. 50 mer Oligonucleotide breakthrough with an Agilent PLRP-S 1000 Å column.

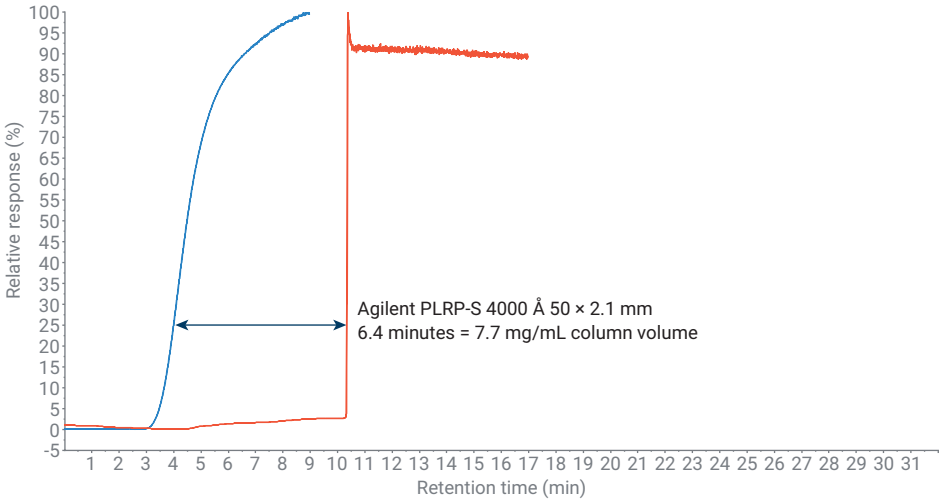
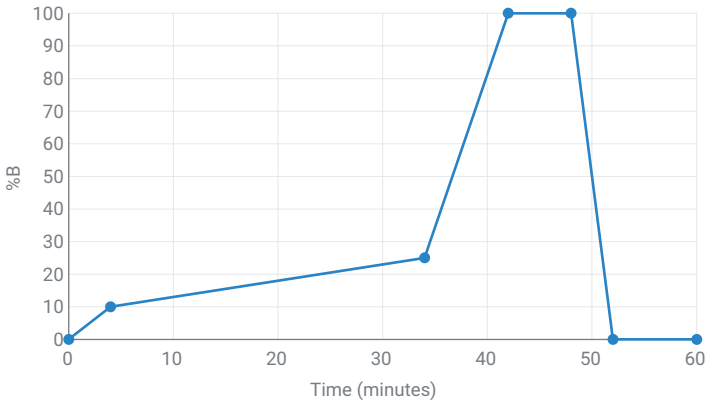


Figure 1D. 50 mer Oligonucleotide breakthrough with an Agilent PLRP-S 4000 Å column.



Time	%A	%B
0	100	0
4	90	10
34	75	25
42	0	100
48	0	100
52	100	0
60	100	0

Figure 2. Gradient profile for column cleanup.

The internal surface area of the stationary phase particles decreases as the pore size increases. It is anticipated that the smaller pore-size PLRP-S 100 Å will have the highest DBC. In practice, this is true for the smallest oligonucleotide, 25 mer (Figure 3). For the larger oligonucleotides (50 and 75 mer) there is a decrease in loading capacity for the PLRP-S 100 Å, as some of the pores become too small.

Taking a closer look at the DBC breakthrough curve for the 50 mer oligonucleotide on the PLRP-S 100 and 300 Å columns, a noticeable change can be seen in the profile, which also indicates the impact that the restricted access to the pores is having on mass transfer (Figure 4). This kind of effect can also be noticed in an analytical separation (Figure 5), where the peak width for the 300 Å pore size column is narrower, indicating better mass transfer.

While the largest pore size 4,000 Å has the lowest surface area, and therefore limited DBC, the large pores allow rapid mass transfer and provide sharp peaks for excellent resolution.

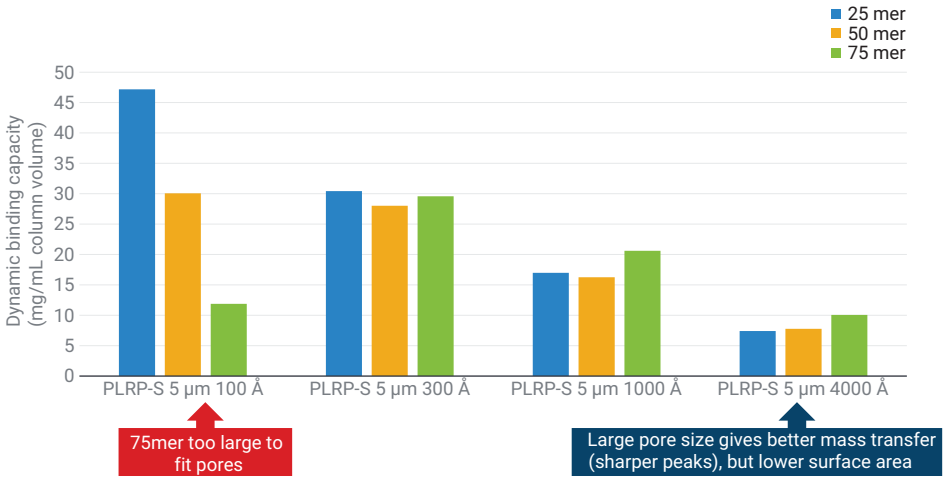


Figure 3. Comparison of binding capacity of different size oligonucleotides.

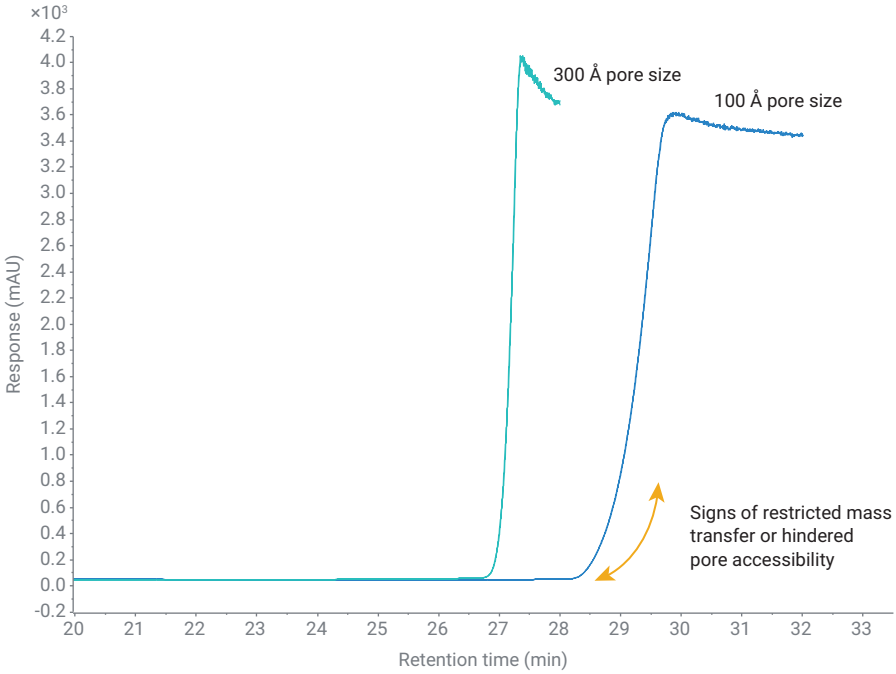


Figure 4. Close up of 50 mer oligonucleotide binding capacity curves of 300 and 100 Å.

Figure 6 shows the analytical separations of the four different crude oligonucleotides, together with a zoomed region to clearly resolve many of the impurities that are present.

DBC provides a useful indication of how a stationary phase may behave for oligonucleotide purification. It can help determine which material may give the best results for purifying a particular oligonucleotide, and give an indication to the column size that may be required. It is not possible to predict the optimum amount of an oligonucleotide that can be purified in a single run, as results will be dependent on the impurities that are present and also the target

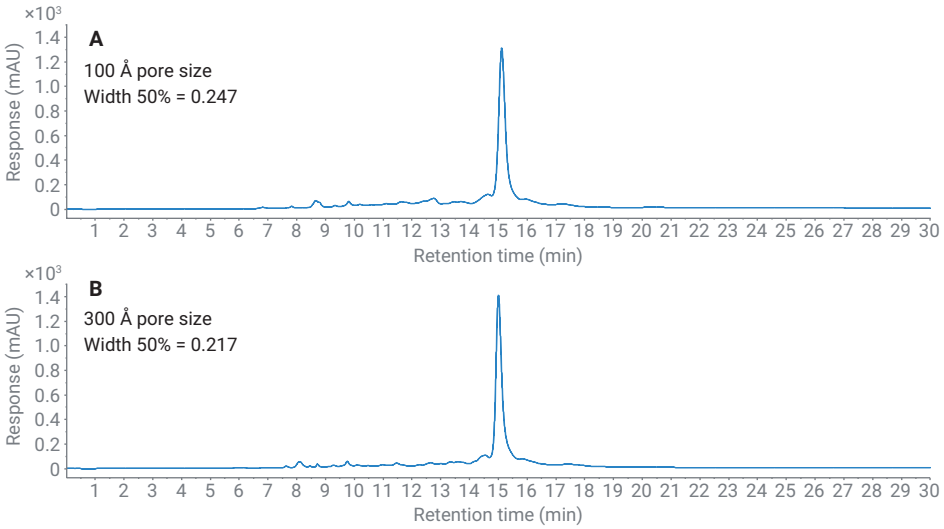


Figure 5. Analytical separation of crude 50 mer oligonucleotide on Agilent PLRP-S 100 and 300 Å columns.

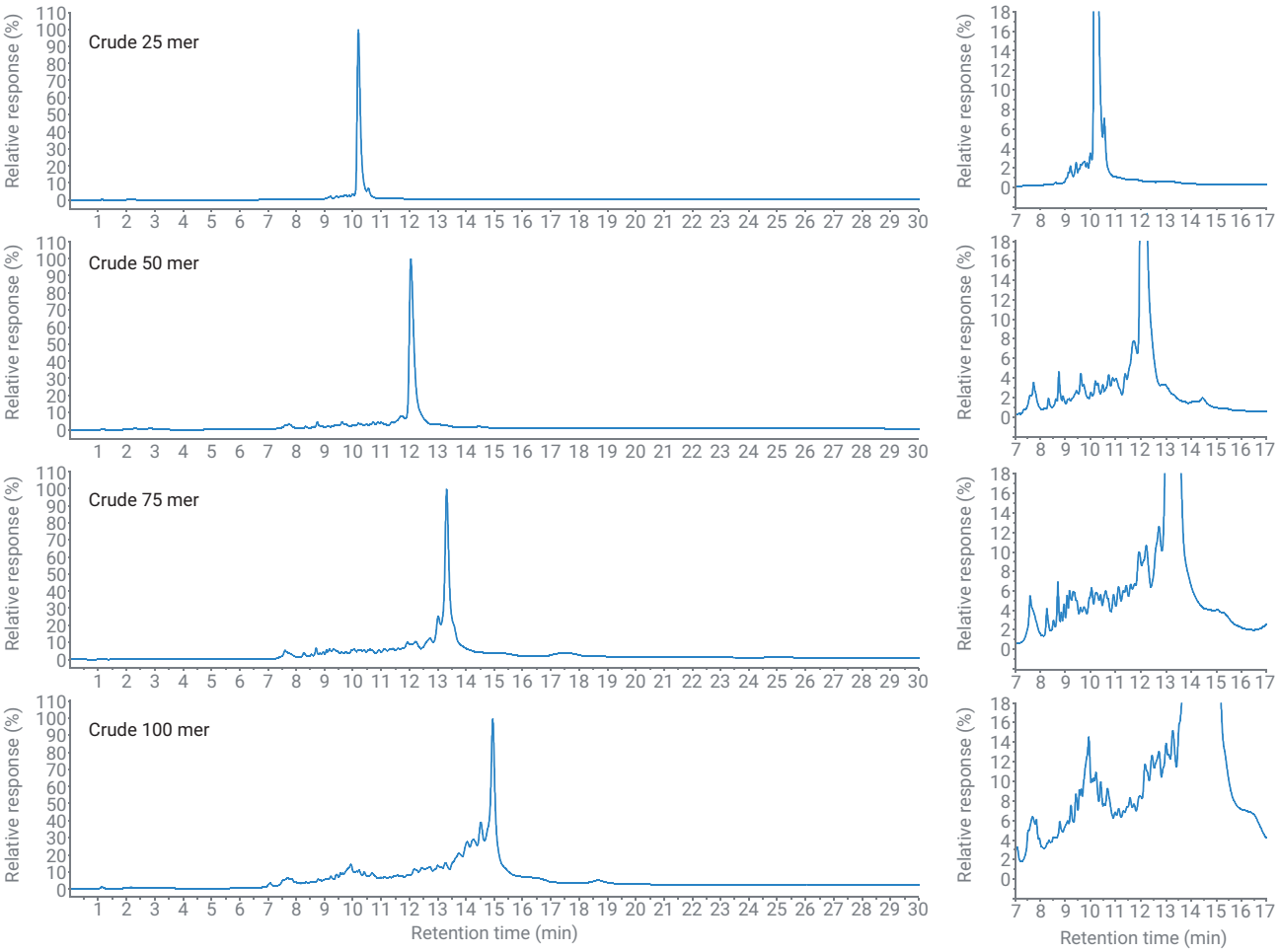


Figure 6. Analytical separation of crude 25, 50, 75, and 100 mer using an Agilent PLRP-S 4000 Å column (left) with zoomed regions (right).

purity. However, using these results as a guideline, helps estimate the amount of oligonucleotide that may be able to be purified as an initial injection quantity from which optimization and validation is performed. For example, using 5% of the DBC as a loading value allows comparison of the potential scale that can be expected for different column diameters and bed depths (Table 4).

Ultimately, it will be necessary to conduct more extensive testing and validation of these figures for each individual purification, to ensure the purity required is achieved. This is best done using an analytical column packed with the same grade stationary phase that will be used for purification.

Conclusion

PLRP-S polymeric materials are an ideal choice for ion pair reversed-phase oligonucleotide purification. They are physically and chemically robust and therefore well suited to the conditions required. Choosing the correct pore size for the length of oligonucleotide is important to optimize the binding capacity and overall oligonucleotide purification.

References

1. Lloyd, L. L. *et al. J. Chromatogr. A* **2003**, *1009*, 223–230.

Table 4. Estimated loading capability by column internal diameter.

		Loading (mg) per 50 mm bed depth (based on 5% capacity)											
		PLRP-S 100 Å			PLRP-S 300 Å			PLRP-S 1000 Å			PLRP-S 4000 Å		
ID (mm)	Vol (mL)	25 mer	50 mer	75 mer	25 mer	50 mer	75 mer	25 mer	50 mer	75 mer	25 mer	50 mer	75 mer
2.1	0.17	0.4	0.3	0.1	0.3	0.2	0.3	0.1	0.1	0.2	0.1	0.1	0.1
4.6	0.83	2.0	1.3	0.5	1.3	1.2	1.2	0.7	0.7	0.9	0.3	0.3	0.4
7.5	2.2	5.2	3.3	1.3	3.4	3.1	3.3	1.9	1.8	2.3	0.8	0.9	1.1
25	24.5	58	37	15	37	34	36	21	20	25	9	10	12
50	98	232	148	58	149	137	145	83	80	101	36	38	50
100	393	927	591	234	597	550	581	334	318	404	145	153	198

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