BDP
Biopharmaceutical Development Program

Standard Operating Procedure

Title: Separation and Quantitation of Plasmid DNA Using Anion-Exchange HPLC with a Tosoh Biosep DNA-NPR Column

SOP Number: 22718 **Revision Number: 02**

Supersedes: Revision 01 **Effective Date:** flEC 6 2015

Originator/Date: [redacted]

Approval/Date:

Approval/Date:

Table of Contents

1.0 Purpose

This procedure describes the use of anion-exchange HPLC with a Tosoh Biosep DNA-NPR column for separation and quantitation of different forms of DNA; open circular (o-DNA), super-coiled (sc-DNA), linear and fragmented forms in plasmid preparations.

2.0 Scope

This procedure applies to Biopharmaceutical Process Analytics\Quality Control (PA\QC) personnel who will perform this procedure. This method is used for qualitative and quantitative separation, quantitation of different forms of DNA (linear, super-coiled, and nicked /open-circled forms) and to estimate the purity of plasmid DNA content. This SOP is applicable for plasmid DNA that is less than ?Kb (Kilobase) in size. Gel-based sc-DNA quantitation may be necessary for plasmids larger than ?Kb (Kilobase).

3.0 Authority and Responsibility

3.1 The Director, Process Analytics\Q uality Control (PA\QC) has the authority to define this procedure.

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract HHSN261200800001E.

3.2 PA\QC is responsible for training laboratory personnel and documenting this training to Biopharmaceutical Quality Assurance (BQA).

3.3 PA\QC personnel are responsible for the performance of this procedure.

3.4 PA\QC is responsible for reviewing the data and documenting the results of this Procedure.

3.5 BQA is responsible for quality oversight of this procedure

4.0 Materials and Equipment

NOTE: Materials and equipment may be substituted with an equivalent grade.

4.1 Quaternary Pump, Agilent Catalog Number G1311A.

4.2 Thermostated Auto sampler, Agilent Catalog Number G1330A and G1329A.

4.3 Photodiode Array Detector, Agilent Catalog Number G1315A.

4.4 Column Compartment, Agilent Catalog Number G1316A.

4.5 Solvent Degasser, Agilent Catalog Number G1322A.

4.6 DNA NPR Column, Tosoh Biosep, 4.6 mm I.D. x 7.5 cm long, 2.5 micron (µm) particle size, BDP PN 21516.

4.7 Guard Column, Tosoh Biosep, 4.6 mm x 0.5 cm, 2.5 micron (µm) particle size, BDP PN 20954.

4.8 Analytical balance.

4.9 Pipetteman and pipette tips (1-200 µL, BDP PN 20469 and 100-1000 µL, BDP PN 20470).

4.10 Glass vial inserts: Agilent, BDP PN 21449.

4.11 2-mL clear, glass HPLC vials, Agilent, BDP PN 20730.

4.12 Sartorious Arium Pro water, HPLC-grade or equivalent.

4.13 Tris base, Sigma, Sigma Ultra Grade, BDP PN 10104.

4.14 AccuGENE 5 M Sodium Chloride, Lonza, BDP PN 30863-1.

4.15 Hydrochloric acid (6N), Fisher, BDP PN 10285.

4.16 Sample dilution buffer (20 mM sodium phosphate 150 mM NaCl pH 7.4, BDP PN 47004).

4.17 pUC 18 plasmid DNA reference standard: Invitrogen, BDP PN 30208.

4.18 Nalgene cellulose nitrate 1- liter filter unit; 0.2 micron (µm) m pore size (BDP PN 20194)

5.0 Buffers and Solutions

5.1 Prepare the following solutions and mobile phases. Record relevant information in the BQC solution logbook (Per ***SOP 22702 - Solutions Used in Process Analytics***, Section 3.0; Form 22702-01) and in the Assay Template, Form 22718-01. Each solution is labeled per ***SOP 22702 -*** **Solutions Used in Process Analytics**, Section 2.3.

5.2 1 M Tris Stock Solution

5.2.1 Dissolve 12.1 g of Tris in 100 mL water in a media bottle. Label the solution's container appropriately.

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NOTE: This solution may be stored at 2-8°C for a month.

5.3 Buffer A (20 mM Tris, 0.5M Sodium Chloride, pH 9.0)

NOTE: Record information in the Assay Template, Form 22718-01 and in a buffer logbook. Assign a lot number that corresponds to the date of preparation.

5.3.1 In a clean 2-liter beaker add the following components in the order specified:

5.3.1.1 20 mL of 1M Tris stock solution

5.3.1.2 100 ml of 5M Sodium Chloride

5.3.1.3 700 mL HPLC water

NOTE: The pH should be 9.00 ± 0.05.

5.3.2 Add 6 M HCl drop-wise using a 100 µL pipetman. Adjust pH to 9.0 ± 0.05. Transfer the contents of the beaker to a 1-liter graduated cylinder. Fill the graduated cylinder to 1 liter with water and mix well. Filter buffer A using a 0.2 µm pore size Nalgene cellulose nitrate 1-liter filter unit. Label the solution's container appropriately (per ***SOP 22702 Solutions used in Process Analytics***, Section 2.3).

5.3.3 Assign a one-week expiration date for Buffer A at room temperature.

5.4 Buffer B (20 mM Tris, 1.0 M Sodium Chloride, pH 9.0)

NOTE: Record information in the Assay Template Form 22718-01 and in a buffer logbook. Assign a lot number that corresponds to the date of preparation.

5.4.1 In a clean 2-liter beaker, add the following components in the order specified.

5.4.2 20 mL of 1M Tris stock solution

5.4.3 200 mL of 5M Sodium Chloride

5.4.4 600 mL water

NOTE: The pH should be 9.00 ± 0.05.

5.4.5 Add 6 M HCl drop-wise using a 100 µL pipettman. Adjust pH to 9.0± 0.05. Transfer the contents of the beaker to a 1-liter graduated cylinder. Fill the graduated cylinder with water to 1 liter and mix well. Filter buffer B using a 0.2 µm pore size Nalgene cellulose nitrate 1-liter filter unit. Label the solution's container appropriately (per ***SOP 22702 - Solutions Used in Process Analytics****,* Section 2.3)

5.4.6 Assign a one-week expiration date for Buffer B at room temperature.

NOTE: Do not use sodium hydroxide to adjust pH when making Tris Buffers

6.0 Procedure

6.1 HPLC Procedure

6.1.1 In the HPLC instrument logbook, enter the QC number, and date of study initiation.

6.1.2 Turn on the computer used to control the Agilent 1100 HPLC. Ensure that the instrument modules are turned on (quaternary or binary pump, DAD, autosampler, column compartment, etc.). If they are not, turn them on.

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6.1.3 Log on to the network using the appropriate username and password specified by the IT Department in the Biopharmaceutical Development Program (BDP).

6.1.4 Open the On-line Software and Method (Refer to ***SOP 22178 - Operation of the Agilent Technologies 1100 HPLC Using ChemStation***, for detailed instructions pertaining to the operation of Agilent 1100 HPLC)

6.1.4.1 Click on the Start menu of the computer.

6.1.4.2 Select “Programs” from the menu.

6.1.4.3 Select “ChemStations.”

6.1.4.4 Choose ChemStation Instrument online.

6.1.4.5 Log on to the ChemStation.

6.1.4.6 Choose the appropriate method (for example: DNANPRQC.M) by selecting the “Methods” from the pull down menu and load method. Select “Edit Entire Method.” Edit the method for accuracy.

6.1.4.7 The HPLC method includes the following parameters.

6.1.4.7.1 Detection: Signal 1 = 254 nm and Signal 2 = 280 nm.

6.1.4.7.2 Column temperature: 25 °C

6.1.4.7.3 Autosampler thermostat: 4 °C

6.1.4.7.4 Stop time: 16.00 minutes.

6.1.4.7.5 Post time: 5.00 minutes.

6.1.4.7.6 Injection volume: 30.0 µL.

6.1.4.7.7 Elution gradient example.

Time (min)	Solv. B	Flow rate (mL/min)	Pressure (Bar)
0.00	0.0	1.000	400
2.00	0.0	1.000	400
7.00	50.0	1.000	400
8.00	0.0	1.000	400
15.00	0.0	1.000	400

6.1.4.7.8 Use the following integration parameters unless specific needs dictate otherwise.

6.1.4.7.8.1 Slope sensitivity: 5.000 (initial)

6.1.4.7.8.2 Peak width: 0.050 (initial)

6.1.4.7.8.3 Area reject: 5.000 (initial)

6.1.4.7.8.4 Height reject: 1.000 (initial)

6.1.4.7.8.5 Initial Shoulders: Off

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6.1.4.7.8.6 Integration: Off (T=0 minutes)

6.1.4.7.8.7 Integration: On (T=2 minutes)

6.1.4.7.8.8 Integration: Off (T=7 minutes)

6.1.4.8 Check buffers and solvents. Click the “ON” button in the on-line window on the right-hand side below the circular GLP symbol. The pump, injector, column thermostat and detector will turn on and turn yellow, then green. When the icons are green, the components are ready.

6.1.4.9 Check to see that the thermostat for the auto sampler is set to 4 °C. To do this, left click with the mouse on the injector and arrow symbol. Click on the thermostat and check to see that it is on and set to 4 °C.

6.1.4.10 The small window below the component window is a plot of absorbance versus time at 280 and 254 nm that can be adjusted by clicking on the crossed arrow icon at the bottom left of the window. The window will open and if the button “adjust” is selected, the signals will be centered on the screen.

6.2 Running Samples and Data Collection

6.2.1 Running samples consists of several steps: generating the HPLC sequence parameters, sequence table, and running the sample(s).

6.2.2 Creating ChemStation Sequence

6.2.2.1 With the “Online” window open and the “Method and Run Control” active, click on the “Sequence” under the main menu.

6.2.2.2 Click on “Sequence Parameters.”

6.2.2.3 Under the data file subdirectory, enter the Quality Control Request Number as the filename. Under parts of method to run according to runtime, select checklist and set wait time at zero. “Nrdy” (Not Ready) timeout should be zero. Click on “ok.”

6.2.2.4 Select “Sequence” from the main menu. Click on “Sequence Table” under Sequence. Create sequence table. The sequence table will show the vial number, sample name, method name, number of injections per vial, and sample type. “Sample Info for vial” is where additional information such as sample concentration, lot number, buffer system, and total sample volume can be entered.

6.2.2.5 Equilibrate the column as follows.

6.2.2.5.1 Buffer A for approximately 1 hour.

6.2.2.5.2 Buffer B for approximately 1 hour.

6.2.2.5.3 Buffer A for approximately 1 hour.

NOTE: Buffers can be run for shorter time periods if baselines appear stable for more than 15-20 minutes.

6.2.2.6 Preparation of Buffer Blank

6.2.2.6.1 Prepare buffer blank by following the steps outlined below.

6.2.2.6.1.1 Place a 200 µL aliquot of Buffer A in an HPLC vial.

6.2.2.6.1.2 Place the vial in the auto-sampler in position 1.

6.2.2.6.1.3 Run six injections of 30 µL aliquots of Buffer A.

NOTE: If the baseline shows excessive noise after six runs, replace the DNA-NPR column with a new one.

6.3 Preparation of DNA Samples, pUC18 Reference Standard, and Sample Dilution Buffer

NOTE: Plasmid DNA samples, pUC18 reference standard, and blank buffer are run after blank buffer A runs are finished and the baseline is determined to be stable.

6.3.1 Prepare pUC18 reference standard by pipetting 40 µL of 0.25 mg/mL pUC 18 into an HPLC sample vial. Add 60 µL of sample dilution buffer (20 mM sodium phosphate 150 mM NaCI pH 7.4) to the same HPLC vial. Mix well. The final concentration of pUC 18 will be 100 µg/mL. Label this vial 'Reference R1". Place in vial position 2.

NOTE: pUC18 reference standard available from vendor at other concentrations can also be used. However, the working concentration of pUC18 reference standard remains at 100 µg/mL.)

6.3.2 Pipette 200 µL of sample dilution buffer into an HPLC sample vial. Label this vial "Blank." Place in vial position 3.

6.3.3 Record the plasmid sample information (given on the QC Test Request Form) on the Assay Template, Form 22718-01. Samples should not be filtered. Dilute all plasmid DNA preparations to the final concentration of 0.1 mg/mL. For example, if DNA sample is initially at 1.0 mg/mL, dilute it 10-fold by adding 225 µL of sample dilution buffer to 25 µL of thawed plasmid DNA. The diluted plasmid DNA is labeled Sample 1 (concentration: 0.1 mg/mL). Record the Initial and diluted concentrations of plasmid DNA in the laboratory notebook and Assay Template, Form 22718-01.

6.3.4 Label 6 HPLC sample vials as S1, S2, S3, S4, S5, and S6. Prepare samples S1 through S 6 by diluting Sample 1 (from Section 6.3) as outlined in Table 1.

Table 1

Sample	Volume of Sample 1 (µL)	Volume of sample dilution buffer (µL)	Final Concentration mg/mL
S1	60	0	0.1000
S2	50	10	0.0833
S3	40	20	0.0666
S4	30	30	0.0500
S5	20	40	0.0330
S6	10	50	0.0166

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6.3.5 Place the vials S1-S6 in the auto-sampler in positions 4 - 9 (S1 in position 4, S2 in position 5, S3 in position 6, S4 in position 7, S5 in position 8, and S6 in position 9).

6.3.6 If more test samples are to be run, prepare sample vials for each and insert in the autosampler and fill up sample table. Each set of six samples should be bracketed by pUC18 reference standard and Blank injections.

6.3.7 Record the information about the sample dilution buffer and the working pUC 18 reference standard in the Assay Template, Form 22718-01

6.4 Data Analysis

6.4.1 The chromatographic profiles of the DNA samples as well as the blank buffer A frequently show a minor peak with an elution time of 0.5-0.6 minutes resulting from protein impurities. This peak should not be included in the integration and DNA purity calculation.

6.4.2 To determine percent of various forms of plasmid DNA present in the sample, use retention time, peak area, and % peak area from chromatograms captured under DAD 254 nm signal.

6.4.3 The software should correctly identify and integrate all peaks. However, in some cases where the peak is very small or does not appear as it should, the computer will not identify and integrate that peak. Therefore, that peak must be manually integrated and documented.

6.4.4 Perform a linear regression analysis for each peak as well as the total peak area using Excel software (or equivalent). Plot the peak area on the Y-axis and the concentration on the X-axis.

6.4.5 Determination of Percent Purity of Plasmid DNA

6.4.5.1 Calculate the percentage of each type of DNA using the following formula.

[Slope of each peak (P1, P2, or P3) ÷ Slope of Total Peak Area] X 100

6.4.5.2 Record the % composition of each plasmid DNA component in the laboratory notebook.

6.4.6 Data Storage

6.4.6.1 The HPLC data is stored on the D:\HPchem\Instrument number\data\ D.

6.5 Reporting Format

6.5.1 Refer to Attachments II and III for a complete example of the report format and supporting data.

6.6 Column Cleaning and Storage

6.6.1 Clean the column with water and then with 30% acetonitrile or 20% methanol after use. Store the column in 30% acetonitrile or 20% methanol when not inuse. Document this process in the instrument logbook

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7.0 Attachments

7.1 **SOP 22178** *Operation of the Agilent Technologies 1100 HPLC Using ChemStation*

7.2 **SOP 22702** *Solutions Used in Process Analytics*

8.0 Attachments

8.1 **Attachment 1** Form 22718-01, Separation and Quantitation of Plasmid DNA using the Anion-Exchanger HPLC with a Tosoh Biosep DNA-NPR Column

8.2 **Attachment 2** DNA-NPR Anion Exchange Column Chromatography Report Format

8.3 **Attachment 3** Determination of Percent Supercoiled Plasmid DNA by IEX-HPLC example

8.4 **Attachment 4** A Representative Chromatogram of pUC 18 Plasmid DNA Screen Shot

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Attachment 1

Separation and Quantitation of Plasmid DNA Using the Anion-Exchanger HPLC with a Tosoh Biosep DNA-NPR Column

NCI-Frederick
Form No.: 22718-01
SOP No.: 22718
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QC Number: _____

Date of Experiment: _____

Column:
DNA-NPR Tosoh Biosep Catalog Number_____Lot Number_____

Guard Column:
Guard-NPR Tosoh Biosep Catalog Number_____Lot Number_____

Buffers:
Buffer preparation documentation: Logbook no._____Page no._____

Buffer A_____pH _____

Buffer B_____pH _____

Date column was first used:_____

Filenames of Buffer A blank runs (file/filename)

1._____ 4. _____

2._____ 5. _____

3._____ 6. _____

Sample Dilution Buffer:_____Lot# _____

pUC 18 standard

Source_____BDP Part no ._____Lot no._____

Preparation of working standard pUC 18

Volume of stock solution_____

Volume of sample dilution buffer_____

Volume of working standard injected_____

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Attachment 1 (Continued)

NCI-Frederick
Form No.: 22718-01
SOP No.: 22718
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Sample Details:

Sample Number _____

Sample Name_____

Lot no._____Concentration_____Storage_____ºC

Sample Preparation:

Indicate Thawing Condition:
Temperature:_____ Thaw Time: _____

Concentration of DNA stock solution _____

Initial concentration of working DNA sample: 0.1 mg/mL

Preparation of working DNA solution:

Volume of sample dilution buffer_____

Volume of DNA Sample (using initial DNA concentration)_____

Sample	Volume DNA (μL)	Volume sample dilution buffer (μL)	Final concentration (mg/mL)	Initial/Date
S1	60	0	0.1000	
S2	50	10	0.0833	
S3	40	20	0.0666	
S4	30	30	0.0500	
S5	20	40	0.0330	
S6	10	50	0.0166	

Performed By:_____ Date: _____

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Attachment 2

DNA-NPR Anion Exchange Column Chromatography Report F**ormat**

BDP Process Analytics (PA\QC) Test Report Summary

QC Test Request Number: QC0XXXXX

Test Article Name / Lot#: XXXXX Plasmid DNA, Lot# XXXXX (Part No. XXXXX)

Study Title: Determination of Percent Supercolied Content of XXXXX Plasmid DNA, Lot# XXXXX by IEX

Study Protocol Number(s): SOP Number 22718 & 22178

Date of Analysis: TBD

Test Result: Supercoiled content of XXXXX Plasmid DNA, Lot# XXXX is XXXX.

I. Assay Description

Plasmid DNA typically exists in super-coiled (sc), relaxed open circular and linear forms. The super-coiled form of plasmid-DNA is the most stable form. Relaxed open circular and linear forms may be generated by stress or enzymatic cleavage of the sc-DNA. The amount of sc-DNA in plasmid DNA preparations is defined as part of product composition as well as part of stability of the plasmid DNA. Ion-exchange HPLC is used to separate and quantitate the different forms of DNA.

II. Procedure

The details of the operation of Agilent HPLC 1100 system used for this analytical procedure are described in the SOP 22178. The procedural details for performing the IEX assay are described in the SOP 22718. A Tosoh Biosep DNA-NPR column (lot # XXXXX, Exp. Date: XX.XX.XX) column was used to perform this analysis. A pUC18 reference standard (Lot# XXXX) was analyzed after each sample.

A linear regression analysis was performed by analyzing the single injections of the test samples at six different concentrations (0.1 mg/mL, 0.0833 mg/mL, 0.0666 mg/mL, 0.05 mg/mL, 0.033 mg/mL, and 0.0166 mg/mL). Percent purity of plasmid DNA was determined. Details of the data analyses results are summarized and shown in Attachments 1-2.

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III. Controls

- **Positive Control(s):**

  XXXXX Plasmid DNA; Reference Standard (Lot# XXXXX)

- **Test Result:**

  Supercoiled Content: XXX%

- **Negative Control(s):**

  Buffer A: 20 mM Tris / 0.5M NaCl, pH 9 (Lot# XXXX; Exp. Date: XXXXX)

IV. Conclusion / Discussion

One sample of XXXX Plasmid DNA and one sample of XXXXX Reference standard plasmid DNA were analyzed by Ion-Exchange chromatography HPLC (IEX) using a Tosoh Biosep DNA-NPR column. The samples were determined to meet the test specification (≥ 75% Super-coiled DNA).

Chromatographic profiles and analyzed results for the test sample XXXXX Plasmid DNA and XXXXX Reference standard Plasmid DNA are included (Attachments XXX).

V. Quality Statement and Approvals

This study has been performed under current Good Manufacturing Practice (cGMP) regulations, as per 21 CFR Part 211, and in accordance with the standard operating procedure(s) listed above. The results obtained have been reviewed for accuracy, completeness, and compliance with established standards. Biopharmaceutical Quality Assurance (BQA) will maintain all original report summaries and associated data for each study performed. Raw data, figures, tables, etc. have been attached to this summary, and meet the requirements for good documentation practices.

Approvals:

Report Prepared By (BQC Personnel)	Date
BQC Supervisor / Director	Date
BQA Representative	Date

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Attachment 3

Determination of Percent Supercoiled Plasmid DNA by IEX-HPLC
SOP 22718

Table 1. Peak Areas and Total Peak Area for XX Plasmid DNA Lot# XX (Part No. X)

Concentration (mg/mL)	Peak 1 Area	Peak 2 Area	Peak 3 Area	Total Peak Area
0.1	27.9	3440	88.2	3556
0.0833	46.5	2910	81.5	3038
0.0666	43.2	2376	49.6	2469
0.05	26.1	1680	27.8	1734
0.033	18.0	1184	21.4	1223
0.0166	12.8	596	10.6	619

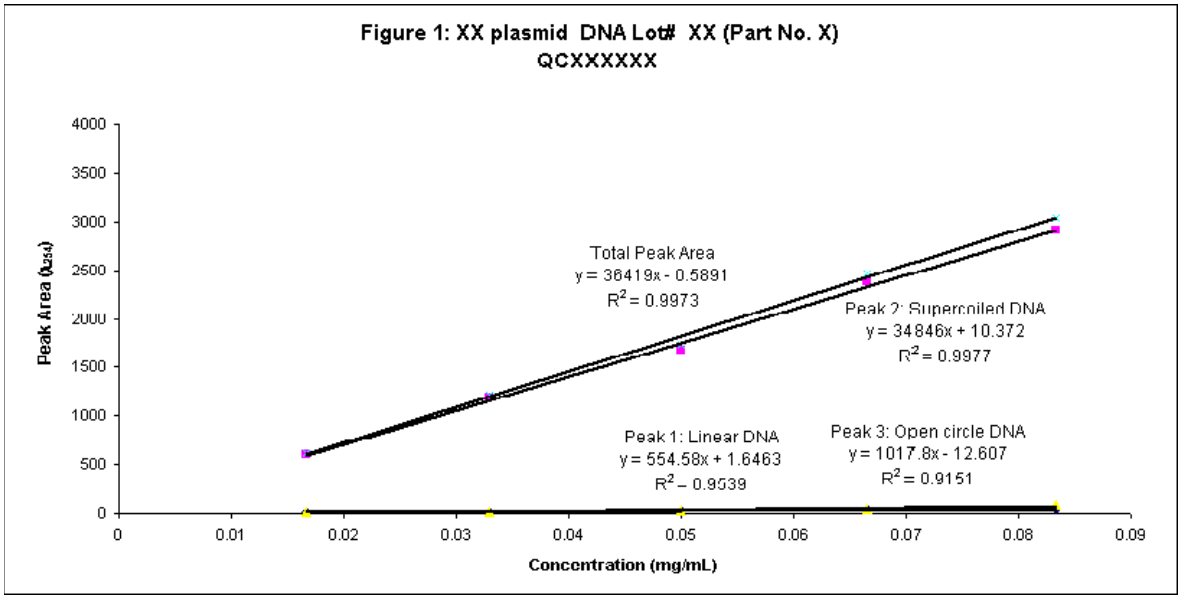

Table 2. Slope and Percent Composition of the DNA Component Peaks of XX Plasmid DNA Lot# XX (Part No. X)

Plot	Component	Intercept	Slope	% of Total	R^2
P1	Linear DNA	1.65	558.6	1.6	0.9539
P2	Supercoiled DNA	10.37	34846	95.7	0.9977
P3	Nicked/Genomic/ Open Circle DNA	12.61	1017.8	2.8	0.9151
PT	Total	0.59	36419	100	0.9973

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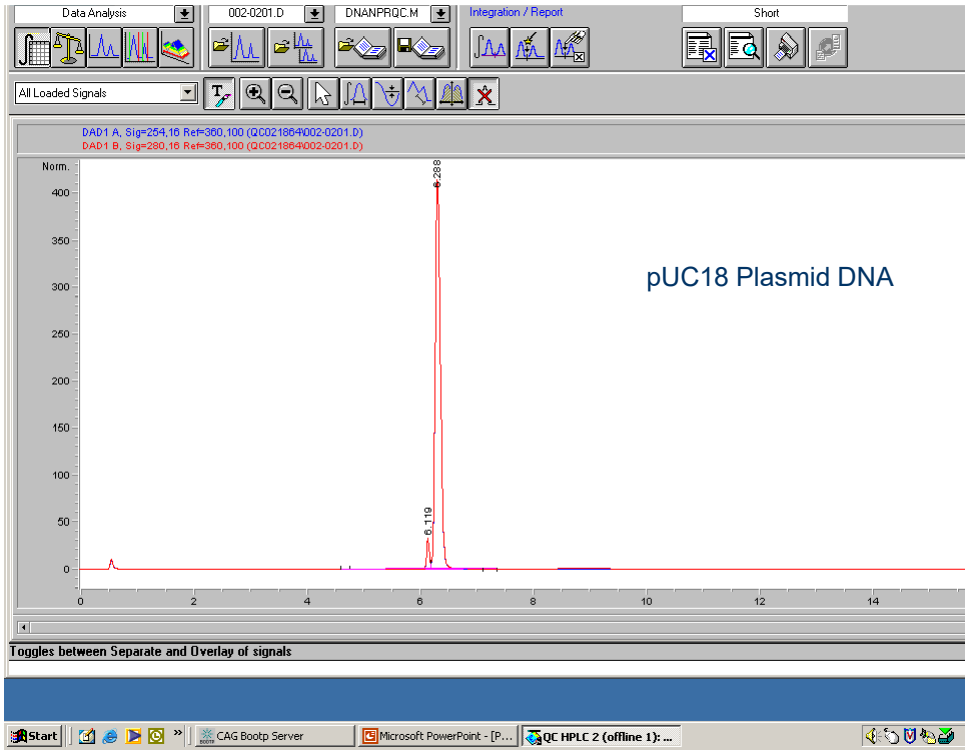
Attachment 4

A Representative Chromatogram of pUC18 Plasmid DNA Screen Shot



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