NUCLEIC ACID THERAPY PURITY METHODS BY CAPILLARY GEL ELECTROPHORESIS

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COVANCE
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Nucleic Acid Therapies

- The number of FDA-approved nucleic acid therapies (NATs) has more than doubled since 2013.
- Both oligonucleotide and gene therapy NATs have been approved.
- mRNA therapies are another type of NAT, and they have shown promise in the clinic.
- Like other biologics, the safety, identity, strength, purity and quality of NAT drug substances and drug products must be determined.
- Capillary Gel Electrophoresis (CGE) can be used to determine NAT Purity.

CGE is Better than Slab-Gel for NAT Purity

- Slab-gels (like agarose) must be manually loaded with sample.
- Nucleic acid sample band migration values (Rf) on slab-gels are determined relative to an internal positive control (like a DNA ladder).
- Percentage of main (NAT) peak and impurity peaks must often be calculated outside the gel imaging software.
- CGE methods use automatic sample injections, sample migration time is measured directly, and percentage of main peak and impurity peaks can be calculated by GMP-compliant software.
- The risk of analyst variability and error is reduced in CGE methods.

NAT Impurities

- Oligonucleotide impurities include base deletions, additions and unremoved protective groups (synthetic failure products).
- Gene therapy impurities include residual host cell and plasmid DNA.
- mRNA therapy impurities include dsRNA and cDNA (plasmid DNA template material).
- The separation of intended NATs from nucleic acid impurities by CGE often depends on the difference in the number of bases (ssDNA) or base pairs (dsDNA) between the therapeutic and the impurity.

Current Study

- NATs vary widely in length from oligonucleotides (less than 100 bases) to gene therapies (up to 4800 base pairs).
- SCIEX PA 800 Plus Pharmaceutical Analysis System was used to separate 20- and 24-base unmodified and phosphorothioate-modified oligonucleotides using the ssDNA 100-R kit.
- The PA 800 instrument was used to separate small plasmid digest fragments (from 500 to 1000 base pairs) using the dsDNA 1000 kit.
- The same instrument was used to separate large plasmid digest fragments (2686 and 4361 base pairs) from each other.

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ssDNA 100-R Kit

- The ssDNA 100-R kit comes with two pre-coated capillaries, lyophilized ssDNA 100-R gel, tris-borate buffer, urea and pd(A) 40-60 test mix.
- The tris-borate buffer was mixed with water and the urea until dissolved, filtered and mixed with the gel to make the gel buffer.
- The capillary was cut to length (30 cm) and filled with gel buffer for conditioning prior to use.
- 0.5 mL of water was used to dissolve the test mix, and enough water for 0.1 mL of 10 OD/mL was added to samples.
- The test mix and samples were electrokinetically injected onto the column and run at 9.3 kV for 40 minutes in reverse polarity mode.
- The capillary temperature was 30ºC and DNA was detected by UV absorbance at 254 nm.

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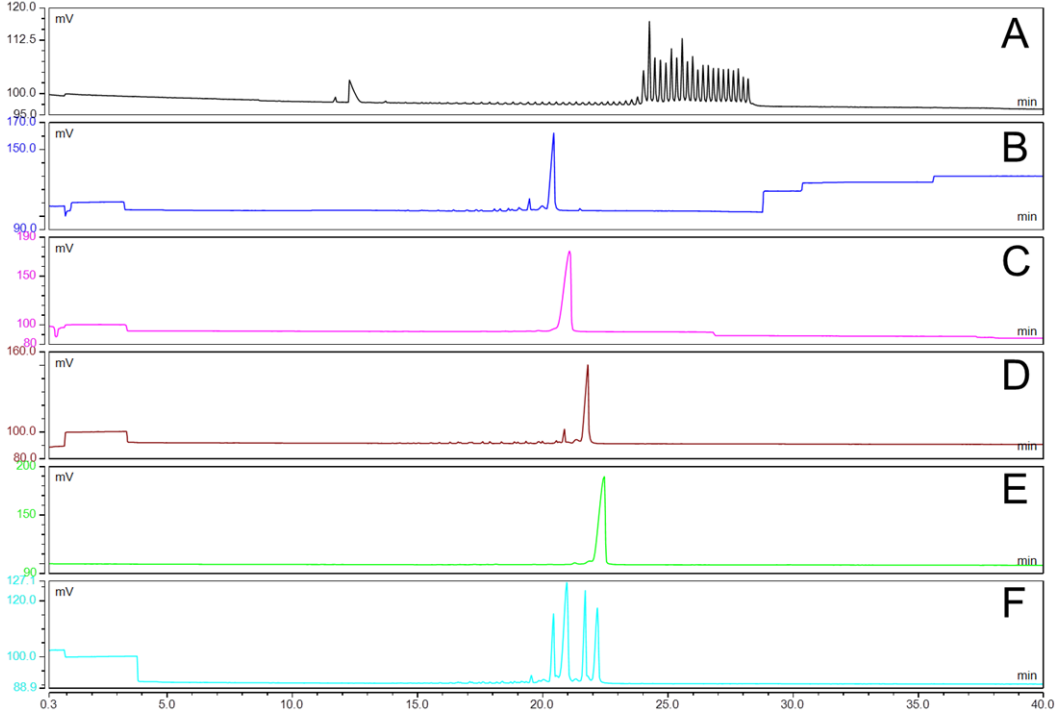
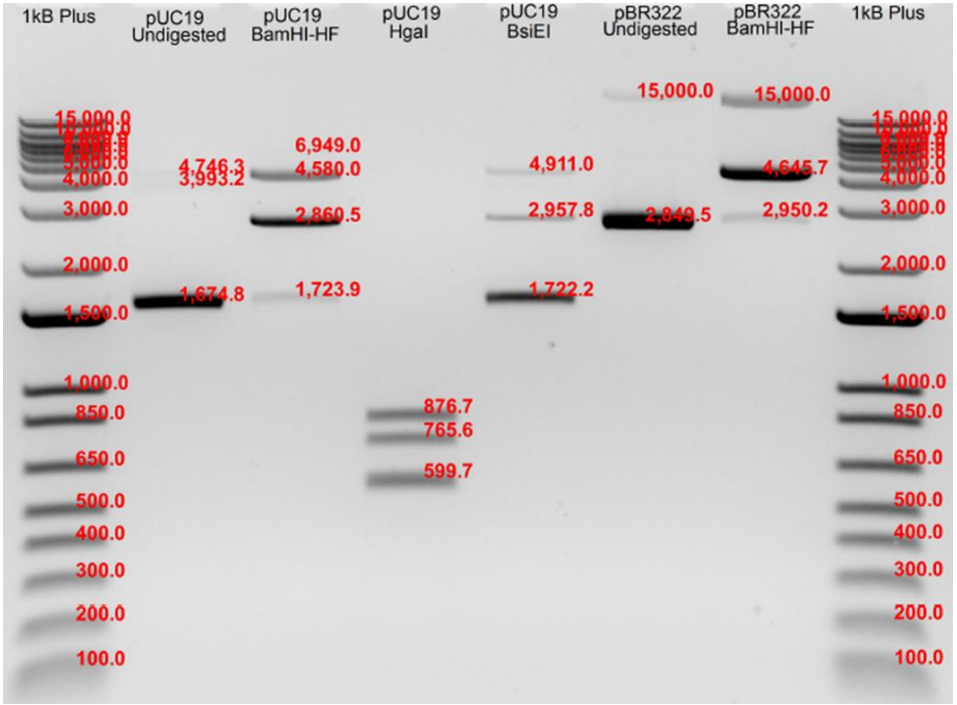
Oligonucleotide Separation with ssDNA 100-R Kit



Data from Covance, Inc.

- pd(A) Test Mix
- 5’-TAATACGACTCACTATAGGG-3’
- 5’-TAATACGACTCACTATAGGG-3’ (Phosphorothioate-Modified)
- 5’-CATACGATTTAGGTGACACTATAG-3’
- 5’-CATACGATTTAGGTGACACTATAG-3’ (Phosphorothioate-Modified)
- Mixture of samples shown in B-E.

Preparation dsDNA Plasmid Fragments for CGE



Data from Covance, Inc.

- 1.2% (w/v) agarose gel stained with ethidium bromide.
- BamHI cuts pUC19 and pBR322 once, whereas HgaI and BsiEI cut pUC19 four and five times, respectively.
- Undigested pUC19 (2686 bp) and pBR322 (4361 bp) mainly in supercoiled form.
- HgaI digestion went to completion, BamHI digestions went mostly to completion, BsiEI digestion was incomplete (fragments not used in CGE).

dsDNA 1000 Kit

- The ssDNA 100-R kit comes with two pre-coated capillaries, dehydrated dsDNA 1000 gel buffer, dsDNA 1000 test mix, and Orange G reference marker (0.1% solution in water).
- The gel buffer was mixed with water until dissolved, filtered and sonicated.
- The capillary was cut to length (30 cm) and filled with gel buffer for conditioning prior to use.
- 40 µL of water and 0.5 µL of Orange G were added to the test mix, and enough water for 0.1 mL of 10 OD/mL, along with 1 µL of Orange G, was added to samples.
- The test mix and samples were electrokinetically injected onto the column and run at 7.8 kV for 25 minutes in reverse polarity mode.
- The capillary temperature is 20ºC and DNA is detected by UV absorbance at 254 nm.

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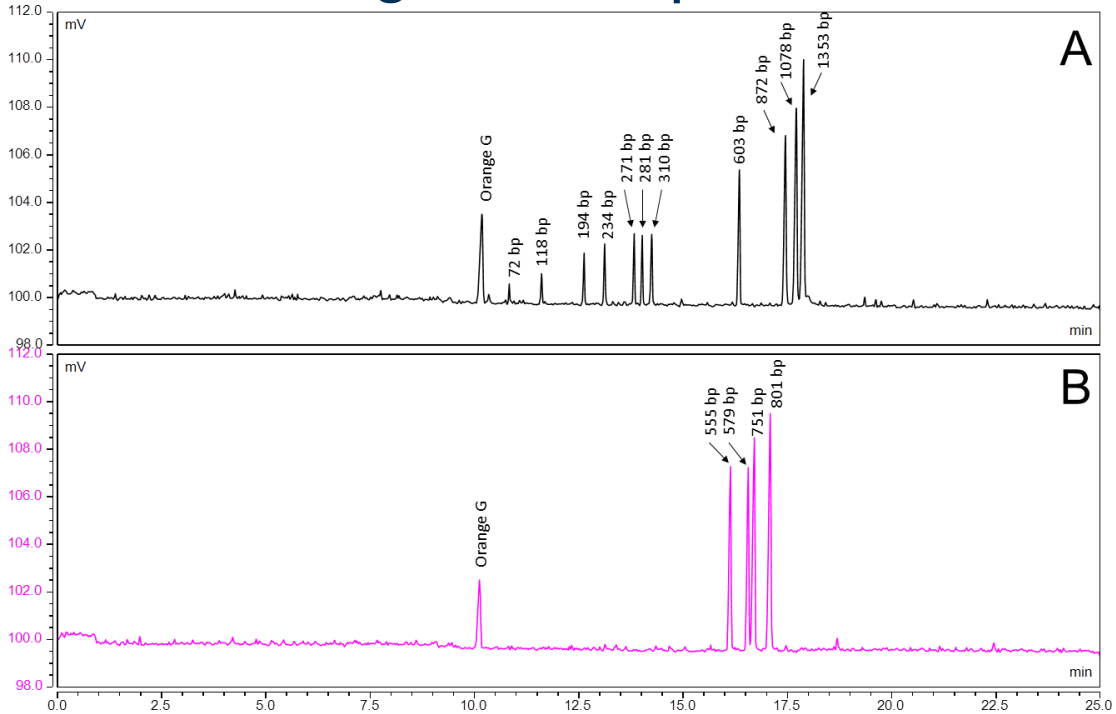
Plasmid Fragment Separation with dsDNA 1000 Kit



Data from Covance, Inc.

- dsDNA 1000 Kit Test Mix (Includes HaeIII Digest of Phi-X 174 and Orange G).
- Digest of pUC19 with HgaI (and Orange G).

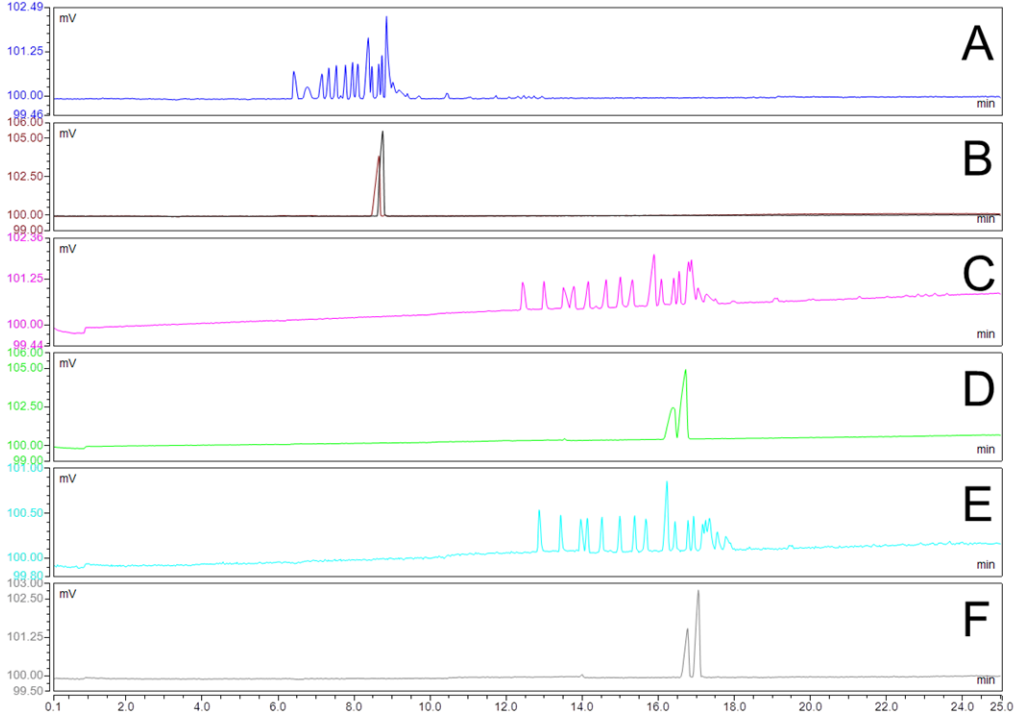
Long dsDNA CGE Method Development

- SCIEX Kit for dsDNA longer than 1000 base pairs not available, so method development was needed.
- A SCIEX Technical note for ssRNA was available (using an uncoated capillary and 1% PVP as the gel buffer).
- Starting with the ssRNA method conditions, the capillary length, pressure, temperature, voltage and %PVP conditions were varied.
- The optimal conditions would be those that give a baseline separation of the BamHI digests of pUC19 and pBR322, and best resolution of 1 kB DNA Ladder peaks, in less than 30 minutes.

Long dsDNA CGE Method Development

- The dsDNA from the pUC19 and pBR322 vectors were digested BamHI and diluted with water to get 0.1 mL of 10 OD/mL. The standard was prepared by adding 2 µL of 1kB Plus DNA ladder to 98 µL water.
- The gel buffer was made by adding 0.5, 1 or 2 grams of polyvinylpyrrolidone (PVP) and 24 grams of urea in 1X tris-borate-EDTA (TBE) to make 100 mL, stirred to dissolve and filtered.
- The uncoated capillary was cut to 30 or 40 cm with or without 20 psi applied to both ends during separation.
- Separations were performed at various voltages (4, 5 or 6 kV) and capillary temperatures (15, 20, 25 or 35ºC) in reverse polarity mode.
- DNA was detected by measuring the UV absorbance at 254 nm.

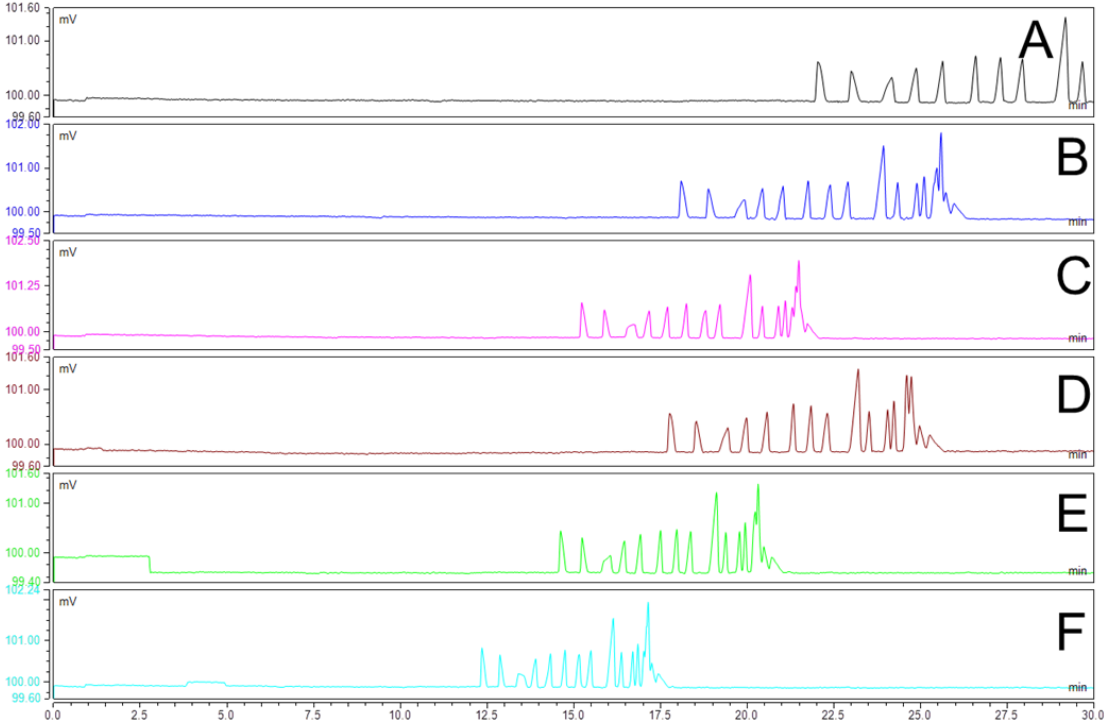
dsDNA CGE MD (Constant: 6 kV, 25°C, 1%PVP)



Data from Covance, Inc.

- 1 kB DNA ladder, 30 cm capillary, 0 psi applied to both ends
- Overlay of pUC19 and pBR322 BamHI digest fragments, 30 cm capillary, 0 psi applied to both ends
- 1 kB DNA ladder, 40 cm capillary, 0 psi applied to both ends
- Mixture of pUC19 and pBR322 BamHI digest fragments, 40 cm capillary, 0 psi applied to both ends
- 1 kB DNA ladder, 40 cm capillary, 20 psi applied to both ends
- Mixture of pUC19 and pBR322 BamHI digest fragments, 40 cm capillary, 20 psi applied to both ends

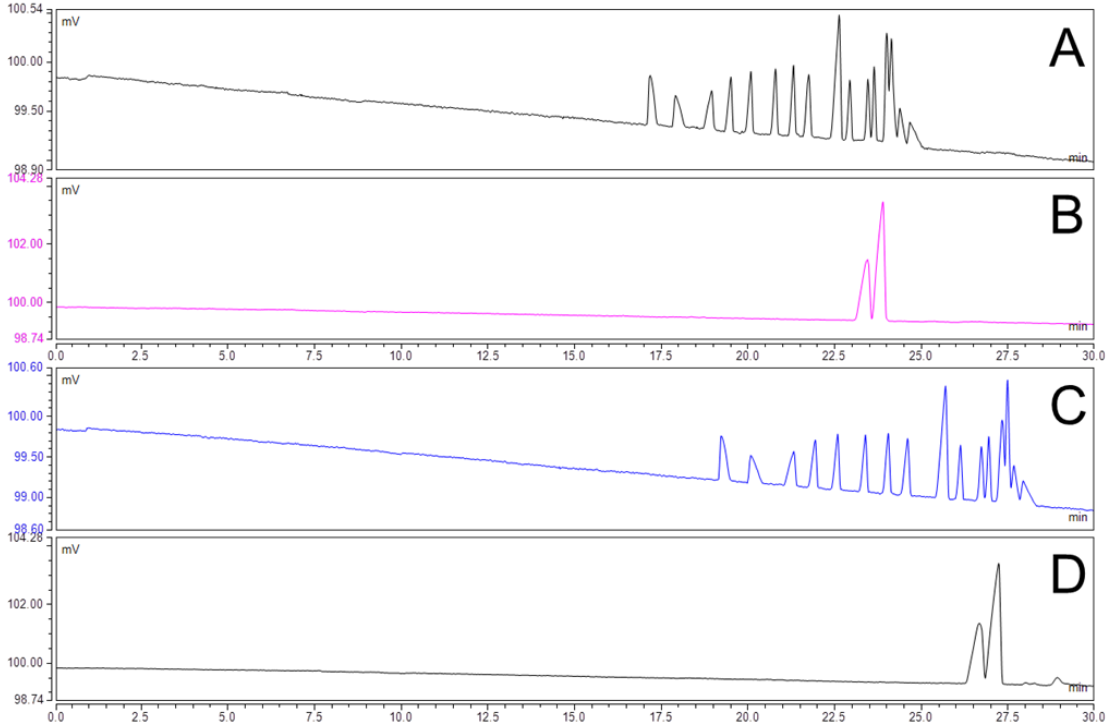
dsDNA CGE MD (Constant: 40 cm, 20 psi, 1%PVP)



Data from Covance, Inc.

- 1 kB DNA ladder, 15ºC, 4 kV
- 1 kB DNA ladder, 25ºC, 4 kV
- 1 kB DNA ladder, 35ºC, 4 kV
- 1 kB DNA ladder, 15ºC, 5 kV
- 1 kB DNA ladder, 25ºC, 5 kV
- 1 kB DNA ladder, 35ºC, 5 kV

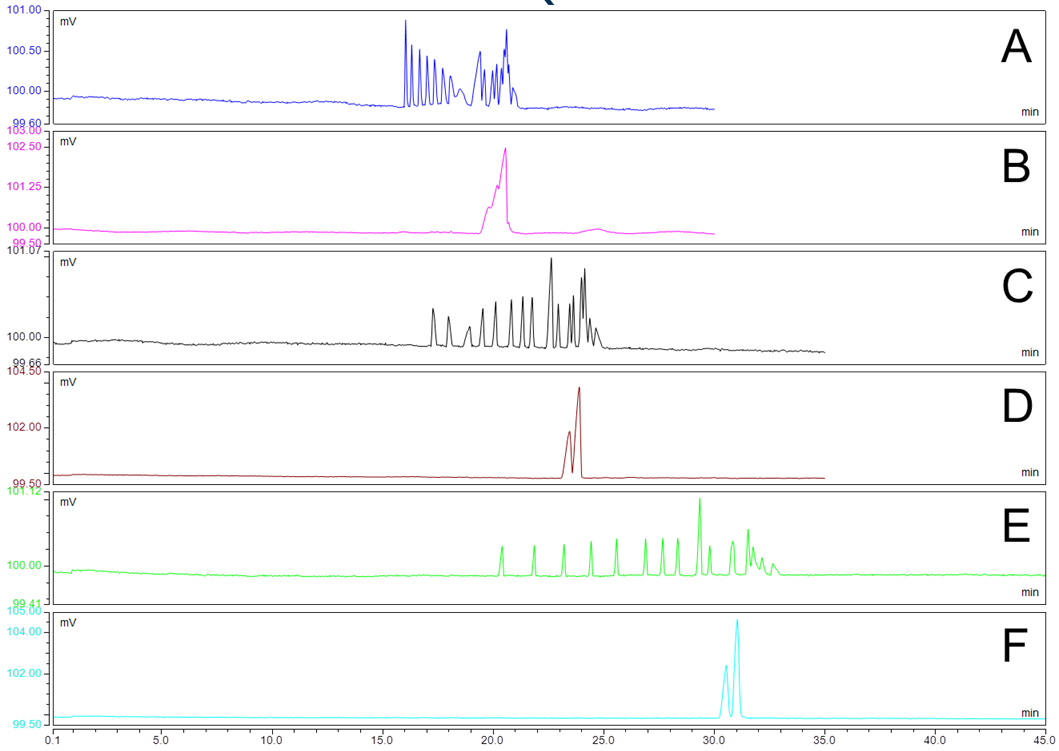
dsDNA CGE MD (Constant: 40 cm, 20 psi, 1%PVP)



Data from Covance, Inc.

- 1 kB DNA ladder, 20ºC, 4 kV
- Mixture of pUC19 and pBR322 BamHI digest fragments, 20ºC, 4 kV
- 1 kB DNA ladder, 15ºC, 5 kV
- Mixture of pUC19 and pBR322 BamHI digest fragments, 15ºC, 5 kV

dsDNA CGE MD (Constant: 5 kV, 15°C, 40 cm, 20 psi)



- 1 kB DNA ladder, 0.5% PVP
- Mixture of pUC19 and pBR322 BamHI digest fragments, 0.5% PVP
- 1 kB DNA ladder, 1% PVP
- Mixture of pUC19 and pBR322 BamHI digest fragments, 1% PVP
- 1 kB DNA ladder, 2% PVP
- Mixture of pUC19 and pBR322 BamHI digest fragments, 2% PVP

Data from Covance, Inc.

dsDNA CGE Method Development Results

- The optimal conditions for separation of linearized dsDNA species were: total capillary length of 40 cm, 15ºC, 20 psi applied to both ends, 5 kV (reverse polarity mode) and 1% PVP gel buffer.
- CGE had better resolution for dsDNA fragments less than 5000 base pairs.
- 1.2% (w/v) agarose gel had better resolution for dsDNA fragments greater than 5000 base pairs.
- However, most NAT gene therapies are less than 5000 base pairs, so the CGE method developed in this study is sufficient to determine purity for NATs in the 1000 to 5000 base pair range.

Conclusions

- Two kit-based CGE methods and one custom-developed CGE method were used.
- Baseline resolution of single-stranded and double-stranded DNA species from 20 bases to approximately 5000 base pairs in length.
- Both major species and minor impurities were observed in the electropherograms.
- CGE is can be used to measure the purity of NATs regardless of length or whether the drug product or drug substance is single- or double-stranded.

Future Directions

- A recent technical note from SCIEX shows improved separation of 5000 and 7000 base pair plasmid isoforms using the dsDNA 1000 kit with a 1:10 dilution of the gel buffer with 1X TBE buffer.
- Future studies could include the separation of BamHI-digested vector DNA samples with the dsDNA 1000 kit (and diluted gel buffer) to compare the performance with the 1% PVP method developed here.
- Other future studies could include the separation of other types of substituted oligonucleotides with the ssDNA 100-R kit, and actual gene therapy drug products using the dsDNA 1000 kit or the 1% PVP method.

Acknowledgements

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Questions

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