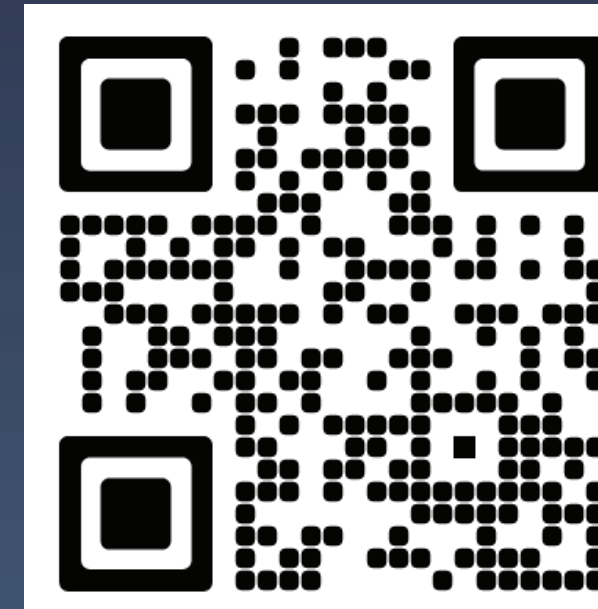




INTRODUCTION

- The Endosomal Escape Vehicle (EEV™) platform was developed to enhance intracellular delivery of oligonucleotides to skeletal and cardiac muscle.¹
- The EEV platform efficiently delivered exon-skipping PMOs to skeletal and cardiac muscle cells in a preclinical model of Duchenne muscular dystrophy,¹ supporting evaluation of their therapeutic potential.
- However, manual synthesis of PMOs is both time-consuming and highly susceptible to human error.²
- The iterative coupling and deprotection cycles of the PMO synthesis process make it amenable to an automated workflow.
 - Automation increases overall speed and efficiency while performing consistent and reliable synthesis.

OBJECTIVE

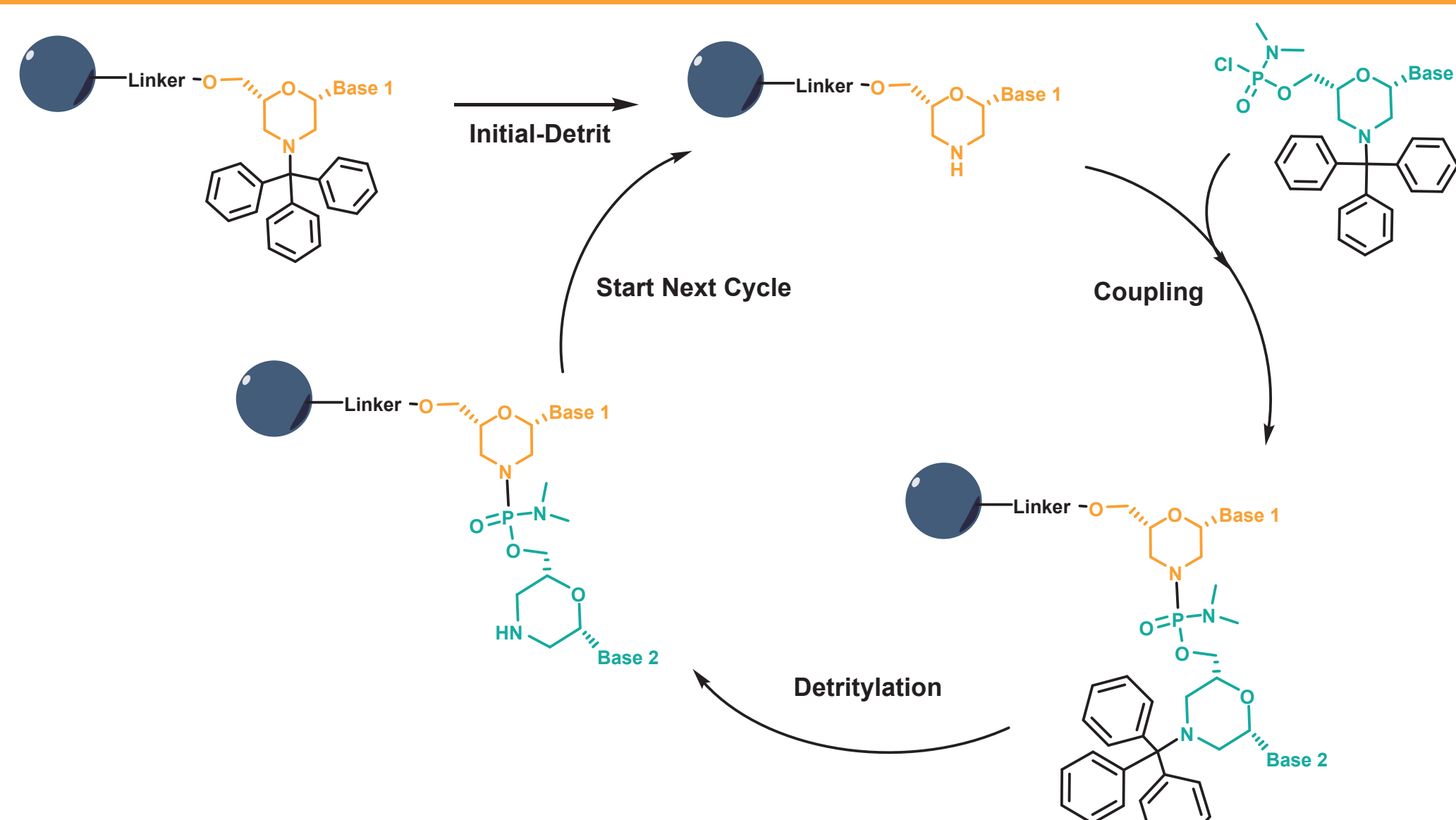
- To adapt a commercially available automated solid-phase peptide synthesizer for synthesizing PMOs.

RESULTS

Steps of PMO Synthesis

- PMO synthesis begins with a resin loaded with PMO monomer and proceeds through iterative cycles of detritylation/deprotection and coupling to generate full-length PMOs (**Figure 1**).
- The manual protocol (**Table 1**) includes in-process controls to confirm efficient detritylation and coupling.

Figure 1. Cycle of PMO Synthesis



Adapted from Li C, et al. *Nat Commun.* 2021;12:4396. doi: 10.1038/s41467-021-24598-4. Published under CC BY license. CyTFA is a detritylation reagent composed of 100 mM trifluoroacetic acid and 100 mM of cyanopyridine in 80:20:1 DCM:TFE:ethanol. The neutralization solution contains 71:24:5 DCM:IPA:DIPEA. The coupling solution contains 0.1 M of PMO monomer and 0.2 M of NEM in DMI. ACN, anhydrous acetonitrile; CyTFA, 4-cyanopyridinium trifluoroacetate; DCM, dichloromethane; DIPEA, diisopropylethylamine; DMI, 1,3-dimethyl-2-imidazolidinone; IPA, isopropanol; NEM, N-ethylmorpholine; PMO, phosphorodiamidate morpholino oligomer; TFE, trifluoroethanol.

Table 1. Manual Synthesis of PMOs on Instrument

Step	Parameter
NMP swelling	2 hours
DCM wash	×3
CyTFA	15 minutes ×3
DCM wash	×3
CyTFA	5 minutes ×1
MSA test	Pass/fail
DCM wash	×3
Neutralization	30 seconds ×2
Neutralization	10 minutes ×1
DCM wash	×3
DMI wash	×1
Coupling	50°C; 2 hours or 30°C; overnight
DCM wash	×3
Chloranil test	Pass/fail

Adapted from Li C, et al. *Nat Commun.* 2021;12:4396. doi: 10.1038/s41467-021-24598-4. Published under CC BY license. CyTFA is a detritylation reagent composed of 100 mM trifluoroacetic acid and 100 mM of cyanopyridine in 80:20:1 DCM:TFE:ethanol. MSA is an in-process control to determine completion of detritylation. The neutralization solution is made up of 71:24:5 DCM:IPA:DIPEA. The coupling solution is 0.1 M of PMO monomer and 0.2 M of NEM in DMI. Chloranil is the in-process control to determine completion of coupling using solutions containing p-chloranil and acetaldehyde. CyTFA, 4-cyanopyridinium trifluoroacetate; DCM, dichloromethane; DIPEA, diisopropylethylamine; DMI, 1,3-dimethyl-2-imidazolidinone; IPA, isopropanol; MSA, methanesulfonic anhydride; NEM, N-ethylmorpholine; NMP, N-methyl-2-pyrrolidone; PMO, phosphorodiamidate morpholino oligomer; TFE, trifluoroethanol.

Automated Peptide Synthesis Instrumentation

- The PurePep Chorus from Gyros was chosen for peptide synthesis because of solvent compatibility and nitrogen-based solvent/reagent delivery (**Figure 2**).
- The instrument accurately dispenses solvents with a range of volatilities commonly used in oligonucleotide synthesis with both top and bottom delivery.
- Each reaction vessel can be mixed via nitrogen bubbling and oscillation to ensure reproducible and uniform reaction protocols.
- The reaction vessels can be heated to improve coupling efficiency.
- The smallest reaction vessel supports synthesis of a scale up to 50 µmol.

Figure 2. Automated Peptide Synthesis Platform

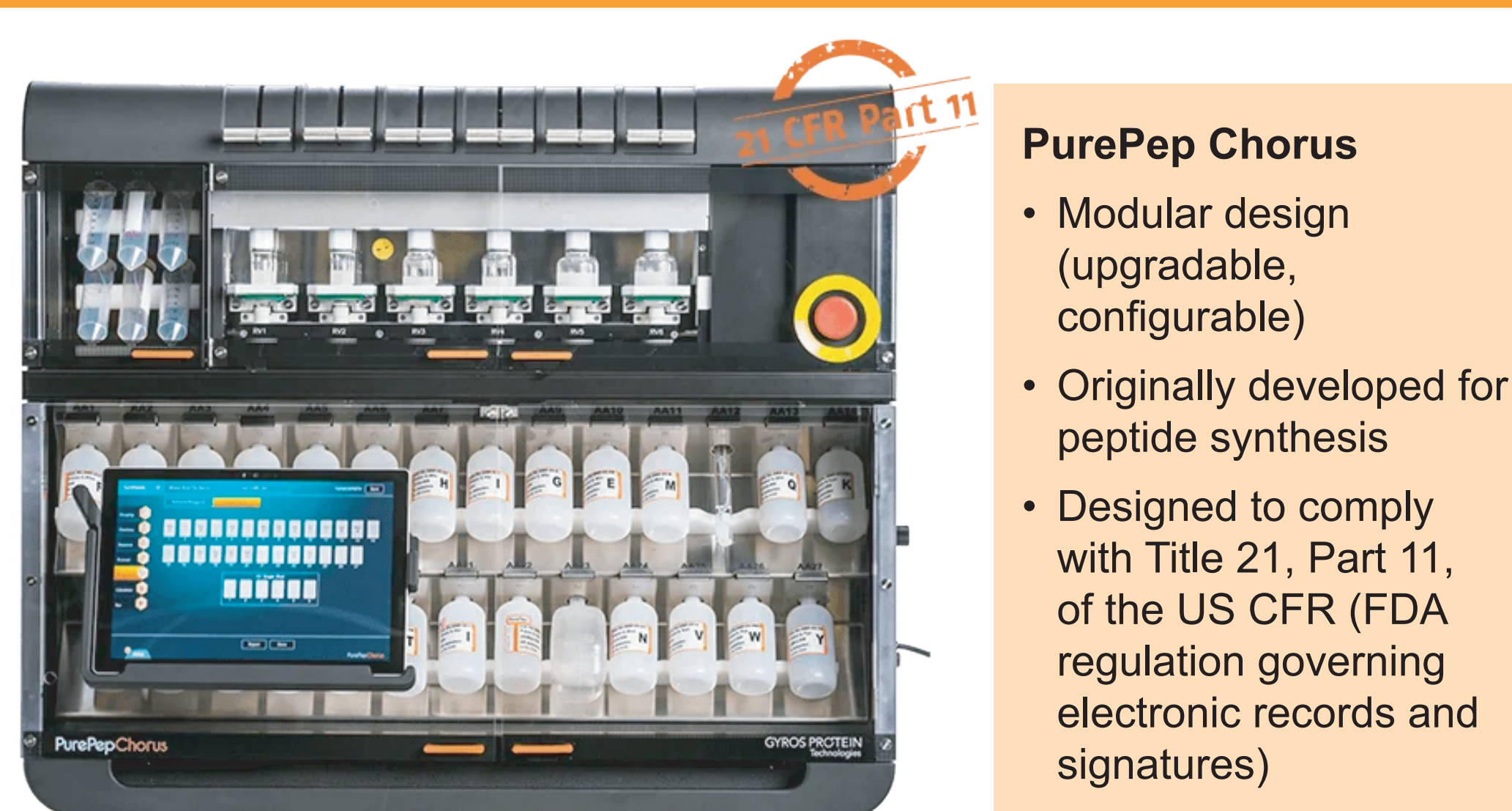


Image: Copyright © 2016 Gyros Protein Technologies AB. All rights reserved. CFR, Code of Federal Regulation; FDA, Food and Drug Administration.

PurePep Chorus

- Modular design (upgradable, configurable)
- Originally developed for peptide synthesis
- Designed to comply with Title 21, Part 11, of the US CFR (FDA regulation governing electronic records and signatures)

Optimized Automated PMO Synthesis

- The steps of optimized automated PMO synthesis are shown in **Table 2**.
- After optimization, there was no resin swelling and fewer detritylation cycles; the resin type and linker were further optimized and longer PMOs were successfully synthesized.

Table 2. Optimized Automatic Synthesis of PMOs on Instrument

Step	Parameter
ACN wash	×3
CyTFA	10 minutes ×2
ACN wash	×2
CyTFA	10 minutes ×2
ACN wash	×2
CyTFA	10 minutes ×2
ACN wash	×3
Neutralization	1 minutes ×3
ACN wash	×3
DMI wash	×1
Coupling	50°C; 2 hours
ACN wash	×6

Adapted from Li C, et al. *Nat Commun.* 2021;12:4396. doi: 10.1038/s41467-021-24598-4. Published under CC BY license. CyTFA is a detritylation reagent composed of 100 mM trifluoroacetic acid and 100 mM of cyanopyridine in 80:20:1 DCM:TFE:ethanol. The neutralization solution contains 71:24:5 DCM:IPA:DIPEA. The coupling solution contains 0.1 M of PMO monomer and 0.2 M of NEM in DMI. ACN, anhydrous acetonitrile; CyTFA, 4-cyanopyridinium trifluoroacetate; DCM, dichloromethane; DIPEA, diisopropylethylamine; DMI, 1,3-dimethyl-2-imidazolidinone; IPA, isopropanol; NEM, N-ethylmorpholine; PMO, phosphorodiamidate morpholino oligomer; TFE, trifluoroethanol.

Manual vs. Automated PMO Synthesis

- Manual (vs. automated) PMO synthesis takes a lot of time, concentration, and effort (**Table 3**).
- Automated synthesis reduced production time for 25-mer PMOs (25-nucleobase oligomer) by ≈3 weeks, minimized error-prone manual steps that can generate N-1 or N+1 products, and helped prevent incorrect-sequence synthesis through consistent execution of predefined programs.
- Non-optimized automated synthesis showed decreasing crude purity relative to manual synthesis as PMO length increased; following process optimization, automated synthesis achieved significantly higher crude purity ($P<0.01$) than manual-synthesis for a full-length 25-mer PMO.

Table 3. Differences Between Manual and Automated PMO Synthesis

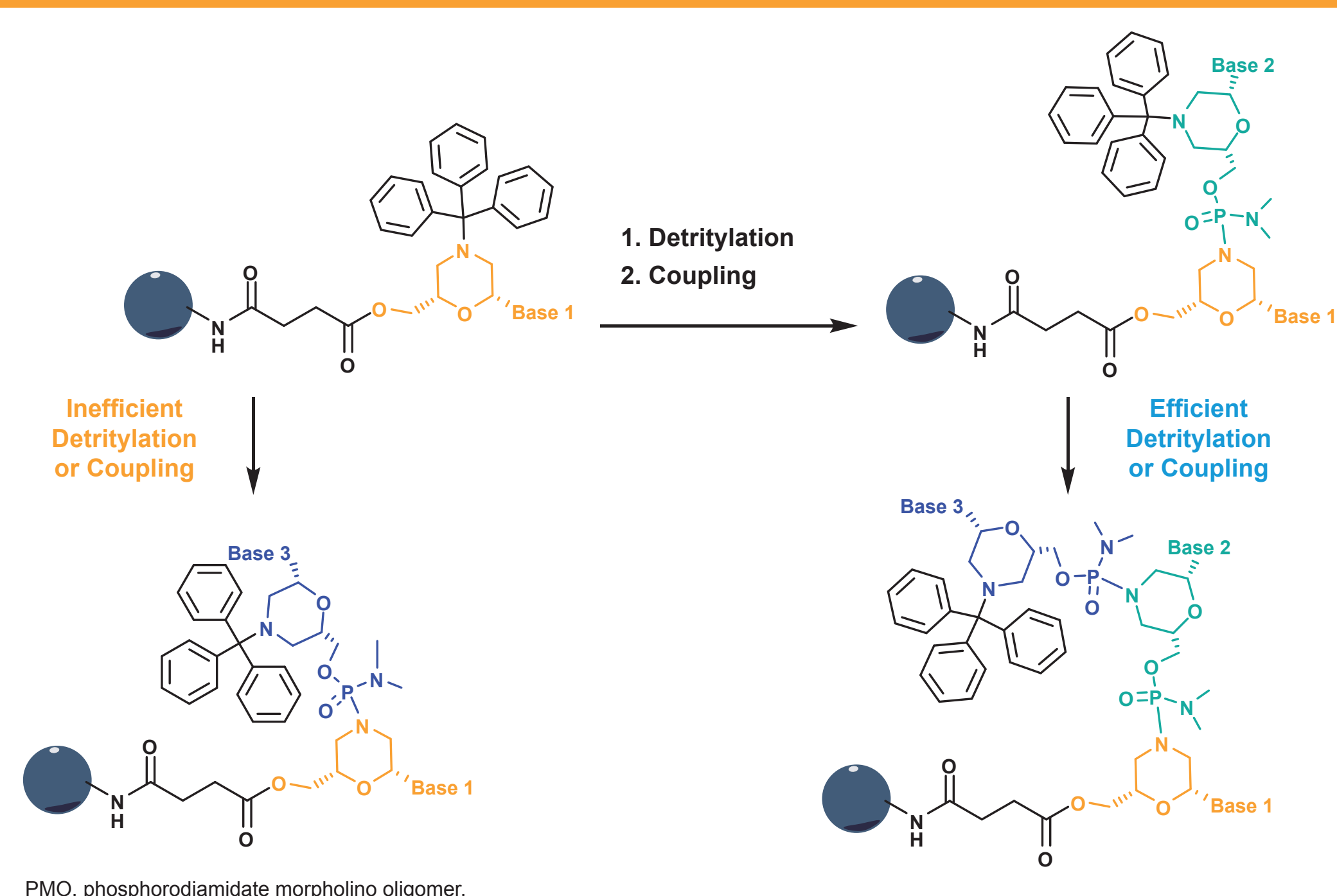
Variable	Manual Synthesis	Automated Synthesis
Total synthesis time	≈1 month	≈1 week
Time spent in the laboratory	≈1 month	≈4 hours
Opportunity for error ^a	Higher	Lower

^aOpportunities for error include 1. N-1: skip a coupling or detritylation step (N-1 product forms and becomes the major product); 2. N+1: perform a coupling step twice without realizing (N+1 forms and becomes the major product); and 3. Incorrect sequence: the wrong PMO monomer is coupled because of misreading or misinterpreting the sequence. PMO, phosphorodiamidate morpholino oligomer.

Formation of N-1 Impurities and Determination of PMO Crude Purity

- Inefficient detritylation or coupling can generate N-1 impurities (i.e., PMOs missing one nucleotide) (**Figure 3**), which are common byproducts in oligonucleotide synthesis.
 - Detritylation:** failure to fully remove the trityl protecting group prevents coupling of the subsequent PMO monomer, causing loss of a nucleobase at that position.
 - Coupling:** failure of monomer addition at some resin-bound PMO chains results in continued synthesis of PMOs lacking one nucleotide.

Figure 3. Formation of N-1 Impurities on Resin During PMO Synthesis



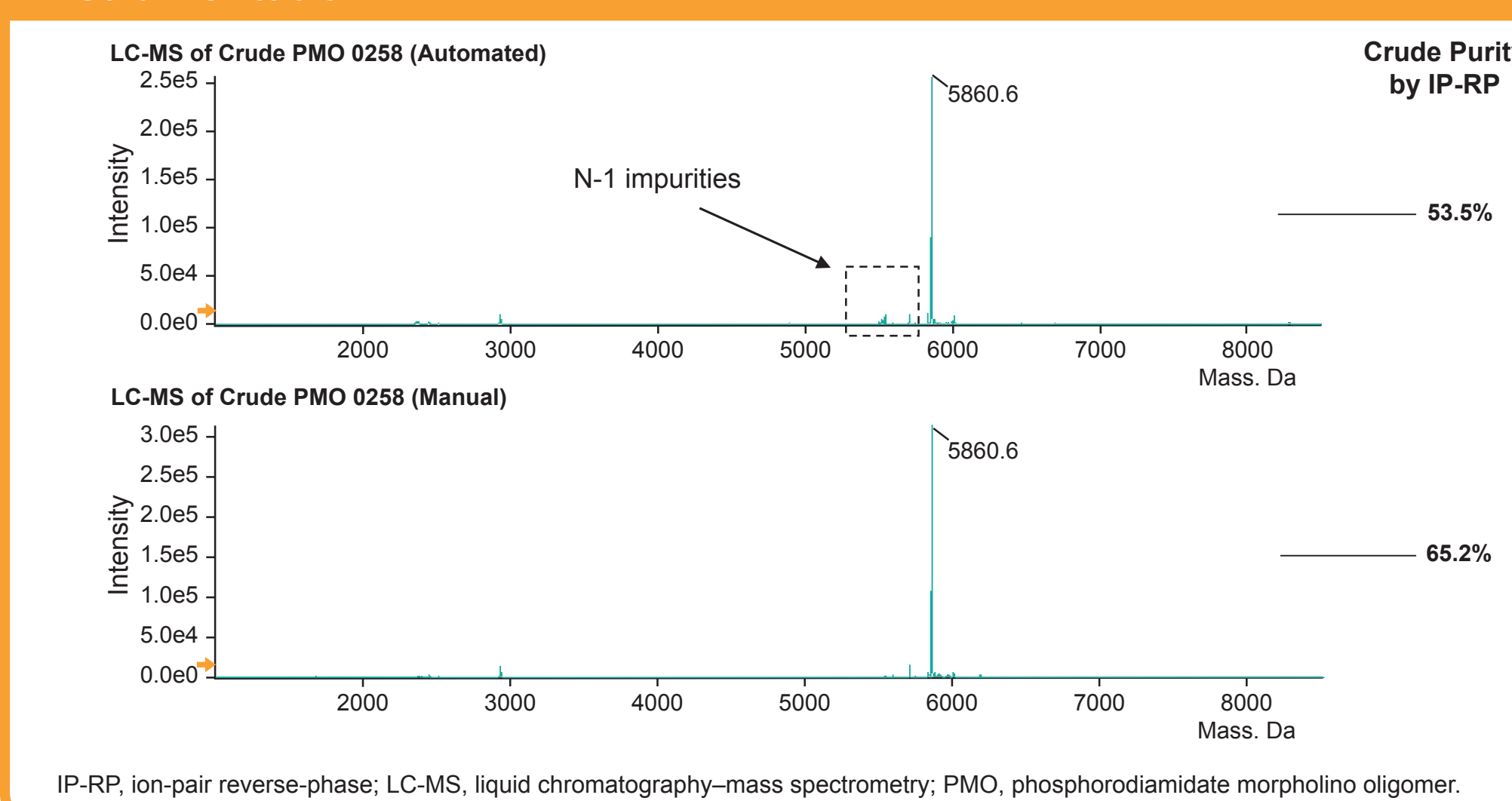
PMO, phosphorodiamidate morpholino oligomer.

MATERIALS AND METHODS

- The PurePep® Chorus (Gyros Protein Technologies, Tucson, AZ) automated peptide synthesizer was used to synthesize PMO sequences.
- This instrument uses positive nitrogen pressure for solvent dispensing and mixing, making it well-suited for dry solvent delivery and maintaining the inert environment required for solid-phase synthesis of PMOs.
- Optimization of synthesis was performed to identify solvents, stoichiometry, and other reaction conditions (e.g., heating, mixing).
- Coupling and deprotection efficiency were evaluated to determine the equivalents of PMO monomer in reaction and the number of detritylation cycles required.
- Two studies were performed with an initial synthesis of six PMO sequences and subsequent synthesis of 18 PMO sequences to test the limitations of the automated methods.

- Crude purity of PMOs synthesized via manual or automated instrumentation was determined by ion-pair reverse-phase chromatography.
- N-1 impurities were identified by liquid chromatography–mass spectrometry (**Figure 4**).
 - N-1 impurities of PMOs generated using the automated peptide synthesizer (vs. the manual method) were 53.3% vs. 65.2% (standard deviation [SD], 7%).
 - Optimized automated synthesis achieved crude purity of PMOs comparable to that achieved with manual synthesis.

Figure 4. Crude Purity of PMOs Generated by Automated or Manual Instrumentation



Coupling and Detritylation Optimization

- Crude purity of full-length 21- to 25-mer PMOs was unaffected by monomer equivalents (3, 5, 7, and 10), coupling conditions, detritylation cycles (four to seven), or post-detritylation wash steps (**Figure 5**).
- Persistent variability suggested that water content was limiting coupling efficiency.
 - The water content in commercially available solvents and reagents was, therefore, assessed by Karl Fischer titration, and molecular sieves were used to further reduce water content.
 - Reducing water content in key synthesis reagents increased crude PMO purity by ≥20%.

Figure 5. Previous Optimization Experiments

Coupling Optimization	
Various equivalents (3 eq, 5 eq, 7 eq, 10 eq)	No difference
Double coupling (2x coupling; 2 hr)	No difference
Increased reaction time (2 hr vs. 4hr)	No difference
Different batches of PMO monomers	No difference
Detritylation Optimization	
Additional detritylation cycles (4x, 5x, 6x, 7x)	No difference
Additional wash sequences for better resin mixing	No difference
Observation	
Variability seems to be related to water content within monomer solutions or solvents	Suggests an issue with coupling reactions

Eq, equivalents; PMO, phosphorodiamidate morpholino oligomer.

Final Purity and Yield of PMO Sequences

- After synthesis of 61 sequences, an average crude purity of ≈59% (SD, 10%), final purity of ≈89% (SD, 3%), and yield of ≈35% (SD, 17%) were achieved.
- With this method, six experimental PMO sequences were synthesized in ≈5 days at a 50-µmol scale.
- This method was also applied to longer 28-mer and 30-mer PMOs, resulting in 50% and 37% crude purity, respectively.

CONCLUSIONS

- Optimization of solvent water content allowed for a consistent and reliable method for generating 21- to 25-mer PMOs.
- This automated method enabled high-throughput and mid-scale (i.e., 50-µmol scale, ≈400 mg by theoretical yield, ≈100–150 mg by final yield) generation of high-purity PMO material.
- The method was successfully utilized to synthesize longer 28-mer and 30-mer PMOs.
- Further optimization of larger reaction vessels may enable scale-up to 100- to 200-µmol synthesis.

ACKNOWLEDGMENTS

Conflicts of interest: all authors are employees of Entrada Therapeutics, Inc. This research was funded by Entrada Therapeutics, Inc (Boston, MA). Editorial and studio support for this poster were provided by Ashfield MedComms (US), an Inizio company, and were funded by Entrada Therapeutics, Inc. References: 1. Li X, et al. *Mol Ther Nucleic Acids.* 2023; 2. Caruthers MH. *Science.* 1985.