FULL-LENGTH ARTICLE

Manufacturing

Small-scale manufacturing of neoantigen-encoding messenger RNA for early-phase clinical trials

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ABSTRACT

Messenger RNA (mRNA) has become a promising tool in therapeutic cancer vaccine strategies. Owing to its flexible design and rapid production, mRNA is an attractive antigen delivery format for cancer vaccines targeting mutated peptides expressed in a tumor—the so-called neoantigens. These neoantigens are rarely shared between patients, and inclusion of these antigens in a vaccine requires the production of individual batches of patient-tailored mRNA. The authors have developed MIDRIX^{NEO}, a personalized mRNA-loaded dendritic cell vaccine targeting tumor neoantigens, which is currently being evaluated in a phase 1 clinical study in lung cancer patients. To facilitate this study, the authors set up a Good Manufacturing Practice (GMP)-compliant production process for the manufacture of small batches of personalized neoantigen-encoding mRNA. In this article, the authors describe the complete mRNA production process and the extensive quality assessment to which the mRNA is subjected. Validation runs have shown that the process delivers mRNA of reproducible, high quality. This process is now successfully applied for the production of neoantigen-encoding mRNA for the clinical evaluation of MIDRIX^{NEO}. To the authors' knowledge, this is the first time that a GMP-based production process of patient-tailored neoantigen mRNA has been described.

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Introduction

In the past decade, messenger RNA (mRNA) has emerged as a promising tool in the field of therapeutic cancer vaccines owing to major improvements in mRNA stability and translatability [1–4]. Several mRNA-based cancer vaccines targeting a wide variety of cancer antigens are currently in clinical development [5]. The most common delivery methods of mRNA cancer vaccines are the direct administration of mRNA with or without a carrier and the indirect application by the

adoptive transfer of *ex vivo* mRNA-transfected dendritic cells (DCs) [6,7]. After cellular uptake, mRNA is translated into a protein and subjected to the patient's own antigen processing machinery, which generates cancer antigens that are presented on HLA molecules to the patient's T cells [5].

The use of mRNA in vaccines combines the advantageous features of a favorable safety profile, intrinsic immunostimulatory capacity and easy manufacturing by *in vitro* transcription (IVT). Furthermore, the sequence of mRNA can be easily altered to encode virtually any epitope. In addition, multiple epitope-encoding sequences can be incorporated into one mRNA construct. The physicochemical properties of mRNA remain largely unaffected by sequence alterations. Therefore, in contrast to other vaccine formats, the same standardized production process can be applied to produce any mRNA construct [8].

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Owing to its adaptability, mRNA is a favorable format for personalized cancer vaccines targeting neoantigens. Neoantigens are mutated peptides that arise as a consequence of non-synonymous somatic mutations in the exome of a tumor. Neoantigen peptides are presented on the HLA molecules of the tumor and can be recognized as foreign by T cells. Cancer mutations are rarely shared between different tumors, and neoantigens are therefore mostly unique to a cancer patient [9]. Consequently, vaccines targeting neoantigens have to be custom-made and manufactured for each individual patient based on the mutanome analysis of the tumor. Personalized neoantigen vaccines with mRNA as an antigen delivery format are now being investigated in phase 1 and 2 studies in several cancer types. A pioneering study by Sahin *et al.* [10] evaluated a naked neoantigen-encoding mRNA vaccine in patients with metastatic melanoma. In a subsequent clinical trial, the investigators evaluated a neoantigen vaccine based on mRNA encapsulated in lipid nanoparticles in patients with triple-negative breast cancer [11]. In a recent report, naked mRNA was administered to patients with gastrointestinal cancer [12]. Together, these trials show encouraging results in terms of safety and immunogenicity but face the critical challenge of timely delivery of the personalized vaccine to the patient.

In most clinical studies, a common workflow is used for the design of neoantigen mRNA. A surgically excised tumor or tumor biopsy and healthy blood sample are collected, and extracted DNA of both samples is subjected to next-generation sequencing to identify the tumor-specific mutations. A bioinformatics pipeline subsequently selects a set of potential immunogenic peptides based on the expression of the mutated gene in the tumor and the predicted binding affinity of the altered peptides to one (or more) of the HLA class I allotypes of the patient [10,12–14]. To further prioritize predicted HLA-binding peptides, the molecular analysis can be supplemented with mass spectrometry (MS)-based proteomics datasets of HLA-eluted peptides from the patient's tumor tissue and/or immunological datasets of T-cell reactivity toward these peptides [14–17]. The prioritized neoantigens are then encoded in the mRNA in a tandem minigene (TMG) configuration, consisting of a string of minigenes, each encoding a selected mutated amino acid flanked on both sides by the amino acids of the wild-type protein [10,12].

Clinical application of mRNA-based neoantigen vaccines in cancer patients requires the production of individual patient-tailored mRNA

batches and timely delivery of the vaccine to the patient. Therefore, the mRNA needs to be manufactured rapidly and on a small scale (1 mg or less). Furthermore, to ensure the safety and efficacy of the vaccine and to adhere to the regulatory requirements of Good Manufacturing Practice (GMP) standards [18], each mRNA batch must be of consistent high quality. This implies that the custom-made mRNA should be produced by a standardized manufacturing process. Custom-made mRNA of acceptable and fully documented quality is, in the authors' experience, difficult to obtain commercially within a short time frame, mainly for logistic reasons.

At the authors' academic institution, MIDRIX^{NEO} was developed, a DC vaccine loaded with neoantigen-encoding TMG mRNA (TMG^{NEO} mRNA), which is currently being evaluated in a phase 1 clinical study in non-small cell lung cancer patients (ClinicalTrials.gov identifier: NCT04078269). To obtain small mRNA batches (about 1 mg) of consistent high quality in a timely manner, the authors set up and validated an in-house mRNA manufacturing process that meets GMP standards. In this article, the authors outline the full manufacturing process and describe the quality tests that are performed to assess the sterility, identity, integrity and purity of the mRNA. The authors further report the results of the validation in which we produced mRNA in three consecutive runs and subjected the resulting mRNA to quality control (QC) and stability testing. To allow for expression analysis and evaluation of immunogenicity, a fluorescent marker or set of immunogenic model epitopes was encoded in the mRNA. The validation mRNA batches passed all quality checks with low batch-to-batch variability. Furthermore, the authors show expression and immunogenicity in antigen-presenting cells, demonstrating that the process allows the production of functional mRNA of reproducible high quality. The manufacturing process is now successfully applied to the small-scale production of patient-tailored mRNA for the clinical evaluation of MIDRIX^{NEO}.

Methods

General overview of the production process

The mRNA manufacturing process comprises several consecutive steps (Figure 1A). In the first step, a patient-tailored DNA plasmid, which encodes a selected set of neoantigens in a tandem of

Figure 1. (A) General overview of the mRNA production process with duration of each manufacturing step. (B) Schematic representation of MIDRIX^{NEO} vaccine manufacturing. (Color version of figure is available online.)

minigenes (TMG^{NEO} plasmid DNA [pDNA]), is generated to serve as a template for IVT of the mRNA. This takes approximately 1 week. The plasmid is then transferred to the GMP facility and subjected to incoming goods QC testing. From this step onward, the process is performed according to GMP standards [18]. Next, the TMG^{NEO} pDNA is linearized and purified. Linearization is a critical step in the mRNA production process, as incomplete linearization can give rise to long, aberrant mRNA transcripts. The linear TMG^{NEO} pDNA is therefore subjected to in-process quality testing. If the linear plasmid meets pre-defined specifications, the process continues to the next step in which mRNA is transcribed by IVT (TMG^{NEO} mRNA). The linear plasmid is subsequently degraded by DNase treatment. Next, the mRNA is precipitated and purified. In the final step, the purified mRNA is filter-sterilized, aliquoted and stored. The mRNA production takes 2 days in total. The final TMG^{NEO} mRNA product is subjected to an array of QC tests to evaluate the sterility, integrity, identity and purity, which takes another 10 days. If the quality of the TMG^{NEO} mRNA complies with all pre-defined specifications, the mRNA is released and used for *ex vivo* loading of autologous monocyte-derived DCs (mo-DCs) to produce the final vaccine, MIDRIX^{NEO} (Figure 1B). The full process, from the generation of the TMG^{NEO} pDNA to the release of the TMG^{NEO} mRNA, takes less than 3 weeks.

Design and construction of a patient-tailored plasmid encoding a TMG of neoantigens

For each patient, TMG^{NEO} pDNA encoding a set of four to six neoantigens is designed and generated. To this end, a TMG encoding up to six patient-specific neoantigens is inserted into a DNA plasmid. This plasmid encodes a T7 promoter upstream of two serial 3' human β -globin untranslated regions and a poly(A) tail of 120 adenosines for improved mRNA stability and translational efficiency in DCs (Figure 2A) [4]. The poly(A) stretch is followed by a unique BspQI (New England Biolabs, Ipswich, MA, USA) restriction site, which is

used for linearization of the plasmid prior to transcription of TMG^{NEO} mRNA. The vector also contains a kanamycin resistance gene for bacterial selection. The plasmid was modified with trafficking sequences for optimized routing to HLA class I and II processing pathways as described by Kreiter *et al.* [3]. More specifically, sequences encoding the signal peptide (SP) and transmembrane and cytosolic domain (major histocompatibility complex class I trafficking domain [MITD]) of HLA-B7 were introduced between the T7 promoter and first 3' β -globin untranslated region sequence as an open reading frame. The SP and MITD sequences were linked by a BamHI (New England Biolabs) restriction site, which is used for in-frame insertion of the TMG^{NEO}.

In the TMG^{NEO}, the neoantigens are encoded in minigenes as 25-mer peptides, which contain the mutated amino acid in the central position flanked bilaterally by 12 amino acids of the wild-type sequence. Up to six minigenes are concatenated and connected by 10-mer glycine–serine linker sequences to create a tandem construct (Figure 2B). The TMG^{NEO} sequence is purchased as a synthetic double-stranded DNA fragment (IDT Technologies, Coralville, IA, USA). In the synthetic DNA fragment, the TMG^{NEO} is flanked by overhangs complementary to the 5' SP and 3' MITD sequences to allow for insertion into the plasmid at the BamHI site by Gibson Assembly (Figure 2C).

To insert the TMG^{NEO}, the plasmid is first linearized by BamHI digestion (New England Biolabs, Ipswich, MA, USA) followed by purification using the Zymoclean large-fragment DNA recovery kit (Zymo Research, Irvine, CA, USA). Subsequently, the linearized plasmid is dephosphorylated with rapid alkaline phosphatase (Roche Life Science, Penzberg, Germany) to prevent self-ligation. The synthetic DNA fragment with complementary overhangs is inserted into the linearized plasmid by Gibson Assembly using the NEBuilder HiFi DNA assembly kit (New England Biolabs). Competent *Escherichia coli* bacteria (New England Biolabs) are transformed with the assembly reaction mix and, as a negative control with dephosphorylated plasmid,

Figure 2. (A) Schematic representation of TMG^{NEO} pDNA. Locations of restriction enzyme recognition sites are indicated. (B) Design of the tandem of minigenes and design of an individual minigene encoding a mutated 25-mer peptide (amino acid sequence is a random sequence). (C) Schema of the insertion of a tandem of minigenes into the pDNA by Gibson Assembly. (D) ORFs of the pDNA templates used for the validation runs and amino acid sequences of the 25-mer peptides encompassing the HLA-A2-restricted model epitopes (underlined). AA, amino acid; dsDNA, double-stranded DNA; GS, glycine–serine; KanaR, kanamycin resistance gene; ORFs, open reading frames; UTR, untranslated region. (Color version of figure is available online.)

plated on lysogeny broth agar (Beckton Dickinson (BD) Difco, Erembodegem, Belgium) plates containing kanamycin antibiotics for selection and grown overnight at 37°C. Individual colonies are selected and grown overnight in liquid lysogeny broth (BD Difco) at 37°C. DNA plasmids are isolated, purified from endotoxins with the Plasmid Plus endotoxin-free midi prep kit (Qiagen, Hilden, Germany) and eluted in water for injection. Presence of the TMG^{NEO} insert and conservation of the full poly(A) tail length are checked by restriction digest and agarose gel electrophoresis (AGE). Plasmid identity is verified by Sanger sequencing (Eurofins Genomics, Luxembourg).

To generate pDNA templates for the validation production runs, the coding sequence of enhanced green fluorescent protein (eGFP) and a TMG encoding three HLA-A2-restricted model epitopes, CMVpp65^{495–503}, gp100^{280–288} and MART-1^{26–35}, are inserted into the SP-MITD open reading frame according to the method described earlier. These plasmids are referred to as eGFP^{VAL} and TMG^{VAL} pDNA, respectively (Figure 2D).

Clean room facility, equipment and quality system

GMP production of TMG^{NEO} mRNA is conducted in a B-grade clean room at the authors' GMP facility (Cell Therapy Unit, Ghent University Hospital). All open production steps are carried out aseptically in a class A biosafety cabinet. The manufacture of the mRNA was designed to reduce the risk of contamination with plasmid DNA/mRNA generated for other patients. For this reason, the biosafety cabinet has an external exhaust to prevent DNA contamination of the grade B environment. To further minimize the risk of cross-contamination, dedicated cleaning agents that have been validated to remove potential DNA and mRNA contaminants are used after each production run and no production runs are performed in parallel. Potential RNases are removed from the cabinet using an RNase decontamination solution (RNase Away; Thermo Fisher Scientific, Waltham, MA, USA). During production, the environment in grade A and B is continuously monitored for viable and non-viable particles. Line clearance is performed before and after every production run. The production is performed by qualified personnel according to standard operating procedures. Each production run is documented in a batch record. All devices are qualified, and storage conditions are monitored and reported.

mRNA production

All raw materials used for mRNA production are provided with a certificate of analysis and certificate of origin by the supplier and checked for potentially hazardous content. Bovine serum albumin-containing materials are certified to be bovine spongiform encephalopathy-free. Where possible, pharmaceutical-grade products are used (see supplementary Table 1). The identity of TMG^{NEO} pDNA is confirmed by Sanger sequencing of the full TMG^{NEO} sequence.

Linearization and purification of pDNA

The TMG^{NEO} pDNA is linearized in individual digestion reactions of 150 µL in 0.2 mL polymerase chain reaction (PCR) tubes (Eppendorf, Hamburg, Germany). Each reaction contains 10 µg TMG^{NEO} pDNA, 100 U BspQI (New England Biolabs, Ipswich, MA, USA), 15 µL 10 × 3.1 buffer (New England Biolabs) and water for injection (B. Braun, Melsungen, Germany) to adjust the final volume to 150 µL. The reaction is incubated in a PCR thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) for 3 h at 50°C and subsequently at 80°C for 20 min to inactivate the reaction. Next, the DNA is purified from the digest using the DNA Clean and Concentrator kit (Zymo Research). In brief, two reactions are pooled in a 1.5-mL microcentrifuge tube (Eppendorf) to which 300 µL of DNA binding buffer per reaction is added. Subsequently, the mixture is loaded on a Zymo-Spin column (Zymo Research) in a collection tube and centrifuged for 30 s at 21

900 g in a tabletop microcentrifuge. The column is washed by adding 200 µL DNA wash buffer and centrifuged for 30 s at 21 900 g. A total of 25 µL of DNA elution buffer is added to the column and incubated at ambient temperature for 5 min. Finally, the column is transferred to a 1.5-mL microcentrifuge tube and centrifuged at 21 900 g for 30 s to elute the TMG^{NEO} pDNA. A sample of the purified linear TMG^{NEO} pDNA is taken for in-process controls. The linear TMG^{NEO} pDNA is stored at –20°C while awaiting the results of the in-process controls and further IVT.

In vitro transcription, precipitation and purification

TMG^{NEO} mRNA transcription is performed using the HiScribe T7 anti-reverse cap analog mRNA kit (New England Biolabs) in individual reactions of 20 µL in 0.2-mL microcentrifuge tubes. For each *in vitro* transcription reaction, 0.5–1 µg pDNA is added to 10 µL 2× anti-reverse cap analog/nucleoside triphosphate mix, 2 µL T7 RNA polymerase mix and water for injection to adjust the final volume to 20 µL. The reaction mix is incubated at 37°C for 2 h in a PCR thermal cycler. To degrade the pDNA, 2 µL DNase I is added to each reaction followed by incubation at 37°C for 15 min. From this step onward, the reactions are continuously held at 4°C and all materials used are pre-cooled. A total of 30 µL water for injection is added to each reaction, and 10 diluted reactions are pooled in a 1.5-mL microcentrifuge tube. To precipitate the mRNA, 250 µL lithium chloride solution is added to each reaction pool and the tubes are stored overnight at –80°C. The following day, the tubes are centrifuged in a pre-cooled tabletop microcentrifuge for 30 min at 21 900 g. Next, the supernatant is removed and the pellets are washed by adding 750 µL of pre-cooled 70% ethanol (96° Sterop diluted with water for injection, Anderlecht, Belgium) and centrifuged for 30 min at 21 900 g. Subsequently, the ethanol is removed and the pellets are air-dried. Finally, each pellet is then resuspended in 200 µL water for injection and the pellets are pooled together.

Sterilization, formulation and storage

Costar Spin-X 0.22-µm centrifuge tube filters (Corning, Corning, NY, USA) are used to sterilize the dissolved mRNA. First, the filters are pre-wet by loading each filter with 100 µL water for injection and centrifuging for 5 min at 21 900 g in a pre-cooled tabletop microcentrifuge. Next, the filters are transferred to a 1.5-mL microcentrifuge tube, whereupon a maximum of 500 µL dissolved mRNA is loaded onto each filter and centrifuged for 5 min at 21 900 g. Finally, the filtered solutions are pooled in a 1.5-mL microcentrifuge tube and mRNA concentration is determined using a NanoDrop Lite (Thermo Fisher Scientific). If the solution is too concentrated, the mRNA is further diluted to the desired final concentration of 1 µg/mL ± 10% with water for injection. In the final step, the TMG^{NEO} mRNA is filled as aliquots of 25 µL in sterile 1-mL cryogenic vials with screw cap (Corning) and stored at –80°C.

In-process QC

Spectrophotometric analysis of linearized TMG^{NEO} pDNA

The concentration and purity of the linearized TMG^{NEO} pDNA are determined by measuring the absorbance (A) of UV light using a Multiskan Sky spectrophotometer (Thermo Fisher Scientific). A_{260nm} is used to determine concentration and the ratio A_{260nm}:A_{280nm} is used to determine purity.

Agarose gel electrophoresis

To confirm linearity, the linearized TMG^{NEO} pDNA is analyzed using AGE. In brief, 300 ng of the linearized pDNA as well as 30 ng and 15 ng of circular plasmid DNA controls is prepared with 6× loading dye and loaded with a TrackIt 1 kb Plus DNA ladder (Thermo Fisher Scientific) on a 1.5% agarose gel stained with Midori Green

DNA stain. The gel is run for 1 h at a constant current of 100 mA. Supercoiled uncut and linear DNA bands are visualized with UV light.

Sanger sequencing

The sequence identity of the linearized TMG^{NEO} pDNA is verified by Sanger sequencing.

QC on the final mRNA

Where available, tests are performed according to the *European Pharmacopoeia 10th edition* (Ph. Eur.). Test methods determining safety (sterility and endotoxin content) were validated, and the other test methods were verified for suitability.

Spectrophotometric analysis of mRNA

Similar to the method used for the linearized TMG^{NEO} pDNA, the concentration and purity of the mRNA are determined by measuring the A of UV light using a Multiskan Sky spectrophotometer. A_{260nm} is used to determine concentration and the ratio A_{260nm}:A_{280nm} is used to determine purity.

Capillary gel electrophoresis

The TMG^{NEO} mRNA is analyzed for size identity and integrity by capillary gel electrophoresis (CGE) with a fragment analyzer and high-sensitivity RNA kit (Agilent Technologies, Santa Clara, CA, USA). The identity of the TMG^{NEO} mRNA is determined by the presence of a single peak at the expected length. In addition, molecular identity is determined by the absence of a peak after RNase digest. TMG^{NEO} mRNA integrity (absence of degradation, presence of full-length mRNA) is assessed by a smear analysis: the area under the curve (AUC) in the interval defined by the peak length $\pm$ 300 bp is determined and expressed as a percentage of AUC the total mRNA.

Sterility testing

Growth of aerobic and anaerobic bacteria as well as fungi is tested according to the Ph. Eur. (chapter 2.6.27). To that end, 100 μ L of mRNA is inoculated in BacT/Alert aerobic iFA and anaerobic iFN hemoculture bottles (bioMérieux, Marcy-l'Étoile, France) and a Sabouraud dextrose agar bottle (Bio-Rad Laboratories). The bottles are cultured for 10 days at 37°C.

Determination of endotoxin content

Determination of endotoxin levels is performed according to the Ph. Eur. (chapter 2.6.14) using the Endosafe-PTS limulus amoebocyte lysate assay (Charles River Laboratories, Wilmington, MA, USA).

Determination of protein content

Determination of residual protein content is performed according to the Ph. Eur. (chapter 2.5.33) using the Micro BCA protein assay kit (Thermo Fisher Scientific).

Determination of residual pDNA content

Residual TMG^{NEO} pDNA plasmid content is determined via quantitative PCR. To limit interference by the mRNA, the quantitative PCR targets a backbone sequence not included in the transcribed sequence.

Potency assays

Expression analysis of eGFP^{VAL} mRNA

A total of 5×10^6 JY cells (lymphoblastoid B-cell line) were resuspended in 170 μ L Opti-MEM medium (Thermo Fisher Scientific). The cell suspension was transferred to a 4-mm electroporation cuvette (Bio-Rad Laboratories), whereupon 5 μ g eGFP^{VAL} mRNA in 30 μ L nuclease-free water was added. As a negative control, solely nuclease-free water was added (mock JY). The cells were electroporated

with an exponential decay pulse of 225 V and 300 μ F in a Gene Pulser Xcell electroporation system (Bio-Rad Laboratories). Immediately after electroporation, cells were transferred to a six-well plate (BD Biosciences, Franklin Lakes, NJ, USA) with 5 mL pre-warmed Iscove's Modified Dulbecco's Medium (IMDM) (Thermo Fisher Scientific) supplemented with 2 mM L-glutamine (Invitrogen, Waltham, MA, USA), 100 IU/mL penicillin (Invitrogen), 100 IU/mL streptomycin (Invitrogen) and 10% fetal calf serum (Invitrogen), hereafter referred to as complete IMDM. After incubation of the plate at 37°C and 5% carbon dioxide (CO₂) for 4 h, eGFP expression in the JY cells was analyzed by flow cytometry.

Immunogenicity assessment of TMG^{VAL} mRNA

Immunogenicity of TMG^{VAL} mRNA was evaluated *in vitro* using two different assays: (i) a chromium-51 (⁵¹Cr) release assay to assess killing of TMG^{VAL}-electroporated JY cells by epitope-specific T cells and (ii) analysis of induction of naive T cells by mo-DCs electroporated with TMG^{VAL} mRNA.

⁵¹Cr release assay to assess killing of a cell line electroporated with TMG^{VAL} mRNA by T-cell receptor-transgenic CD8⁺ T cells. Killing of JY cells electroporated with TMG^{VAL} mRNA by CD8⁺ T cell lines expressing a transgenic HLA-A2-restricted T-cell receptor (TCR) specific for CMVpp65⁴⁹⁵⁻⁵⁰³, MART-1²⁶⁻³⁵ and gp100²⁸⁰⁻²⁸⁸ was assessed in a ⁵¹Cr release assay. In brief, JY cells were transfected with TMG^{VAL} mRNA or irrelevant antigen mRNA (eGFP mRNA) as described earlier and left to rest for 16 h at 37°C and 5% CO₂. As positive controls, JY cells were pulsed for 16 h at 37°C and 5% CO₂ with the relevant peptides by adding these peptides to 1×10^6 JY cells in 1 mL complete IMDM at a final concentration of 1 μ g/mL. After incubation, 0.5×10^6 mRNA-transfected or peptide-pulsed JY cells were labeled with 50 μ Ci ⁵¹Cr (PerkinElmer, Waltham, MA, USA), washed and added at 10^3 cells per well to a 96-well V-bottom plate (Thermo Fisher Scientific). The TCR-transgenic and, as an additional control, non-transgenic T-cell lines were added in ratios ranging from 20:1 to 1:1. The plate was incubated at 37°C and 5% CO₂ for 4 h followed by measurement of the amount of released radioactivity in the supernatant using the 1450 LSC & Luminescence Counter (PerkinElmer), and % specific lysis was calculated using the following formula: (experimental release – spontaneous release) / (maximum release – spontaneous release) $\times$ 100.

Induction of naive CD8⁺ T cells by mo-DCs electroporated with TMG^{VAL} mRNA. To assess the capability of TMG^{VAL} mRNA-electroporated mo-DCs to induce antigen-specific naive T cells, the authors set up a co-culture of mo-DCs with autologous naive CD8⁺ T cells from a healthy HLA-A2⁺ blood donor. As HLA-A2⁺ donors have high frequencies of naive MART-1 CD8⁺ T cells, the authors assessed the expansion of MART-1-specific T cells after stimulation with TMG^{VAL} mRNA-electroporated mo-DCs [19]. The mo-DCs were generated using the authors' 4-day protocol as previously described [20]. At day 4, the mature mo-DCs were electroporated with TMG^{VAL} mRNA or irrelevant antigen mRNA (eGFP mRNA), left to rest for 4 h and cryopreserved. Naive CD8⁺ T cells were purified as previously described and cryopreserved. On the starting day of the co-culture (day 0), the mo-DCs and CD8⁺ T cells were thawed and left to rest for 1 h. Non-electroporated DCs were pulsed with MART-1²⁶⁻³⁵ peptide (GenScript, Piscataway, NJ, USA) for 1 h. The mRNA-electroporated and peptide-pulsed mo-DCs were co-incubated with the CD8⁺ T cells at a 1:10 ratio in Roswell Park Memorial Institute–GlutaMAX medium (Invitrogen) supplemented with 2 mM L-glutamine, 100 IU/mL penicillin, 100 IU/mL streptomycin and 10% fetal calf serum. At day 3 and day 10, 20 ng/mL recombinant human IL-2 (R&D Systems, Minneapolis, MN, USA) was added. At day 7, the autologous CD8⁺ T cells were restimulated with the corresponding mo-DCs. At day 14, the cells were stained with phycoerythrin-conjugated A*02:01/human MART-

Table 1

Results of in-process controls on linearized pDNA intermediate of validation and clinical batches.

Test		Method	Specification	Validation batches			Clinical batch
				Batch 1 (eGFP ^{VAL} mRNA)	Batch 2 (eGFP ^{VAL} mRNA)	Batch 3 (TMG ^{VAL} mRNA)	Batch 4 (TMG ^{NEO} mRNA)
Identity	Concentration	Spectrophotometric analysis: A ₂₆₀	≥125 ng/μL	246 ng/μL	206 ng/μL	304 ng/μL	524 ng/μL
	Sequence	Sanger sequencing	Sequence identical to theoretical sequence	Conforms	Conforms	Conforms	Conforms
Purity	Nucleic acid purity	Spectrophotometric analysis: A ₂₆₀ :A ₂₈₀ ratio	1.7–1.9	1.80	1.80	1.83	1.81
Linearity	AGE		One visible band at theoretical height	Conforms	Conforms	Conforms	Conforms
			Absence of bands of circular plasmids				

1 major histocompatibility complex pentamer (Sanquin, Amsterdam, the Netherlands).

Analysis of translational efficiency of TMG^{NEO} mRNA by MS-based proteomics

The MIDRIX^{NEO} mo-DCs were lysed by probe sonication in a buffer containing 8 M urea, and extracted proteins were reduced, alkylated and digested with endoproteinase Lys-C and trypsin as described previously [21]. Resulting peptides were C18-purified and injected for analysis on an UltiMate 3000 RSLCnano chromatography system online coupled to a Q Exactive HF mass spectrometer (LC-MS/MS) analysis on an UltiMate 3000 RSLCnano chromatography system coupled to a Q Exactive high-frequency mass spectrometer (Thermo Fisher Scientific). Raw spectral data were searched against the

TMG^{NEO} mRNA-encoded amino acid sequence combined with the UniProt human reference database (20 621 entries as of June 2020) using the MaxQuant software package [22].

Results

Validation of the mRNA production process

Production and QC of three validation batches

To ensure that the production process allows reproducible manufacturing of small mRNA batches of high quality, the authors performed a process validation by producing mRNA in three consecutive pilot runs and subjecting the produced batches to a quality assessment. The quality was evaluated in terms of identity, integrity,

Table 2

Results of release controls on final mRNA of validation and clinical batches.

Test		Method	Specification	Validation batches			Clinical batch
				Batch 1 (eGFP ^{VAL} mRNA)	Batch 2 (eGFP ^{VAL} mRNA)	Batch 3 (TMG ^{VAL} mRNA)	Batch 4 (TMG ^{NEO} mRNA)
Yield		Spectrophotometric analysis: concentration × total volume	N/A	0.25 mg	0.50 mg	1.22 mg	1.06 mg
Identity	Concentration	Spectrophotometric analysis: A ₂₆₀	1000 ng/μL ± 10%	967.5 ng/μL	1001.3 ng/μL	1052.5 ng/μL	1032.6 ng/μL
	Length	CGE: peak length	One peak at calculated size ± 20%	Conforms	Conforms	Conforms	Conforms
Integrity	Molecular identity	CGE after RNase digestion	No detectable mRNA peak	Conforms	Conforms	Conforms	Conforms
		CGE: % mRNA in interval peak length ± 300 bp	≥70%	79.7%	80.7%	74.4%	79.4%
Purity/Impurities	Sterility	BacT/Alert	No growth of aerobic or anaerobic bacteria or fungi	Conforms	Conforms	Conforms	Conforms
	Endotoxin content	LAL assay: LOD 0.00125 EU/μg	<0.025 EU/μg mRNA	<0.00125 EU/μg	<0.00125 EU/μg	<0.00125 EU/μg	<0.00125 EU/μg
	Nucleic acid purity	Spectrophotometric analysis: A ₂₆₀ :A ₂₈₀ ratio	2.0–2.3	2.16	2.17	2.23	2.20
	Residual proteins	Micro BCA assay: LOD 2.5 μg/mL	<10 μg/mL mRNA; <1% of mRNA	4.69 μg/mL	<2.5 μg/mL	<2.5 μg/mL	2.88 μg/mL
	Residual DNA	qPCR: LOD 0.01 μg/mL	<0.5 μg/mL mRNA; <0.05% of mRNA	<0.01 μg/mL	<0.01 μg/mL	0.02 μg/mL	<0.01 μg/mL
Potency	Protein expression	Flow cytometry analysis	Detectable expression	Conforms	Conforms	N/A	N/A
	Immunogenicity	T-cell assay	Induction of antigen-specific T cells	N/A	N/A	Conforms	N/A
	Protein Expression	Shotgun mass spectrometry	Detection of at least one peptide derived from the TMG ^{NEO} mRNA-encoded protein	N/A	N/A	N/A	Conforms
Accepted?		N/A	Complies with all specifications	Yes	Yes	Yes	Yes

BCA, bicinchoninic acid; LAL, limulus amoebocyte lysate; LOD, limit of detection; N/A, not applicable; qPCR, quantitative PCR.

sterility, purity and potency. To make an initial assessment of the quality of the mRNA, the authors first performed two runs in which mRNA was produced with a minimum batch size (0.25 mg) to cover for QC assays. As a surrogate, the authors used pDNA encoding eGFP (eGFP^{VAL} pDNA) as a template for IVT. This allowed the authors to easily assess the potency of the final eGFP-encoding mRNA by flow cytometry expression analysis. To be representative of clinical runs, the third run was performed using pDNA encoding a TMG of three HLA-A2-restricted model epitopes (TMG^{VAL} pDNA). Here the batch size was set to 1 mg, which is sufficient to cover QCs and to produce five doses (250 million DCs in total) of the MIDRIX^{NEO} vaccine product for the complete treatment of one patient.

The results of the in-process controls of the linearized pDNA of the three validation batches are shown in Table 1. Linearized pDNA was visualized by AGE, and purity was measured by spectrophotometric analysis. AGE showed linearity of the DNA, with less than 5% contaminating circular plasmids in all three batches. Spectrophotometric analysis demonstrated high purity of the linear pDNA, as $A_{260\text{nm}}:A_{280\text{nm}}$ ratios ranged from 1.80 to 1.83.

The results of the QC on the final mRNA of the three batches are shown in Table 2. The batches were of high purity, as demonstrated by $A_{260\text{nm}}:A_{280\text{nm}}$ ratios ranging from 2.16 to 2.23 and levels of residual protein and DNA that were below or near the limit of detection. Safety was determined in terms of microbiological contamination by performing sterility and bacterial endotoxin testing.

All three batches had undetectable endotoxin levels and were sterile, as no growth of fungi or anaerobic or aerobic bacteria was observed. Identity and integrity were analyzed by CGE, which showed that the vast majority of the mRNA transcripts of all three batches had the expected length and only limited amounts of degraded/aberrant transcripts were present, as quantified by a smear analysis (Figure 3A). Absence of the mRNA peak on CGE after RNase digestion of the three batches confirmed the molecular identity of the mRNA (data not shown). Collectively, the results of the final QC testing on the three batches complied with the pre-defined specifications (Table 2). In conclusion, the manufacturing process consistently delivers mRNA that meets quality specifications with low batch-to-batch variability.

Storage stability of validation batches

Storage stability at -80°C was tested on the three mRNA batches at several time points up to 1 year. As partial degradation of mRNA by possible contaminating RNases is the major determinant of stability, the authors analyzed the integrity of the mRNA by CGE (smear analysis: % of mRNA in peak interval) as a proxy of stability. All three batches proved to be stable up to at least 1 year, as there was no substantial increase in degraded transcripts and smear analysis results conformed to specifications ($\geq 70\%$) (Figure 3B). Based on these results, the shelf life of the mRNA at -80°C was determined to be 1

Figure 3. (A) Length and integrity analysis of validation and clinical mRNA batches. Electropherograms generated by CGE show the length in nucleotides (nt) in the x-axis and RFUs in the y-axis. Integrity was evaluated by smear analysis, with area under the curve in the interval determined by peak length ± 300 nt expressed as a percentage of the total area. These percentages are indicated on the electropherograms as %peak interval (PI), and the intervals are demarcated by dashed lines. (B) Stability of mRNA in the validation batches. Stability was determined by repeating integrity analysis by CGE after 3 months, 9 months and 12 months of storage at -80°C . Dashed line indicates the minimal smear analysis value (70%) needed to conform to integrity specifications. nt, length in nucleotides; RFUs, relative fluorescence units. (Color version of figure is available online.)

Figure 4. Potency assays of validation mRNA batches. (A) Expression of eGFP^{VAL} mRNA in JY cells. JY cells were electroporated with mRNA of batch 1 and 2, and eGFP expression (green histogram) was evaluated 4 h later by flow cytometry. JY cells electroporated with water (gray histogram) were used as negative controls. (B) Immunogenicity of TMG^{VAL} mRNA in JY cells. Killing of JY cells electroporated with TMG^{VAL} mRNA of batch 3 by the TCR-transgenic CD8⁺ T-cell lines was evaluated in a ⁵¹Cr release assay. JY cells and T cells were co-incubated for 4 h in decreasing E:T ratios. As controls, eGFP mRNA-electroporated and peptide-pulsed JY cells were used. Shown values are the means of duplicate determinations, with error bars indicating the standard error of the mean. (C) Immunogenicity of TMG^{VAL} mRNA in mo-DCs. Flow cytometry dot plots show MART-1 pentamer staining in CD8⁺ T cells at day 0 and day 14 of co-culture with autologous mo-DCs electroporated with TMG^{VAL} mRNA of batch 3 and as controls with non-peptide-pulsed, MART-1 peptide-pulsed and eGFP mRNA-electroporated mo-DCs. Plots shown are representative of triplicate determinations. EP, electroporated; E:T, effector:target; NTD, non-transduced; PP, peptide-pulsed. (Color version of figure is available online.)

year, which is amply sufficient to perform QC testing and production of the final MIDRIX^{NEO} vaccine product.

Potency of validation batches

To evaluate the biological functionality of the produced mRNA, the eGFP^{VAL} mRNA of batch 1 and batch 2 and TMG^{VAL} mRNA of batch 3 were tested for expression and immunogenicity, respectively. The eGFP^{VAL} mRNA was electroporated in JY cells and expression was evaluated using flow cytometry. Both batches showed eGFP expression in JY cells, indicating that the mRNA was successfully translated into protein (Figure 4A). Immunogenicity of TMG^{VAL} mRNA was assessed in two ways. First, the authors evaluated whether the mRNA allowed for proper processing and presentation of each single HLA-A2-restricted epitope encoded in the TMG. The mRNA was electroporated in HLA-A2⁺ JY cells, and the killing of these JY cells by CD8⁺ T-cell lines expressing a transgenic HLA-A2-restricted TCR specific for one of the three epitopes (MART-1, CMVpp65 and gp100 TCR) was evaluated. By performing a ⁵¹Cr release assay, the authors showed that the TMG^{VAL} mRNA-electroporated JY cells were efficiently killed by each of the three TCR-transgenic T-cell lines, demonstrating that the TMG mRNA allowed for appropriate antigen presentation of each of the encoded epitopes (Figure 4B). Second, the authors evaluated whether the antigens encoded in the TMG^{VAL} were efficiently presented by mo-DCs, which represent the final vaccine format. Mo-DCs were produced from the peripheral blood mononuclear cells of a healthy HLA-A2⁺ leukapheresis donor according to the authors' previously described

clinical protocol. As HLA-A2⁺ donors have high frequencies of MART-1-specific naive CD8⁺ T cells detectable by flow cytometry, the authors were able to assess antigen presentation by evaluating autologous MART-1 T-cell expansion after long-term co-culture with TMG^{VAL} mRNA-electroporated DCs. Mo-DCs were electroporated with TMG^{VAL} mRNA and incubated with naive CD8⁺ T cells from the same donor for 14 days. T-cell expansion was evaluated by flow cytometry detection of MART-1 pentamer-positive T cells at day 0 and day 14. The authors were able to show that almost no MART-1-specific T cells could be detected after stimulation with mo-DCs electroporated with irrelevant antigen mRNA (eGFP mRNA), whereas the TMG^{VAL} mRNA-electroporated DCs induced a 36-fold expansion of MART-1 epitope-specific T cells (Figure 4C). This indicated that the mo-DCs efficiently presented the antigens encoded in the TMG mRNA.

Clinical production

The validated mRNA production process is currently being applied in the clinical production of TMG^{NEO} mRNA as a component of the MIDRIX^{NEO} vaccine. The results of the in-process and release QC assays of a representative TMG^{NEO} mRNA batch for clinical use are shown in Tables 1 and 2. In the clinical setting, potency testing of the mRNA is performed at the final MIDRIX^{NEO} drug product level. The TMG^{NEO} mRNA-transfected DCs were subjected to a whole proteome analysis by LC-MS/MS to verify whether the TMG^{NEO} mRNA was successfully translated. In the MIDRIX^{NEO} DCs transfected with the TMG^{NEO} mRNA,

Figure 5. Potency assay of clinical TMG^{NEO} mRNA batch. (A) Amino acid sequence encoded in the TMG^{NEO} mRNA construct. Stretches marked with turquoise bars indicate peptide sequences detected by MS. (B) Abundance ranking of all cellular proteins quantified by LC-MS/MS using iBAQ [23]. The TMG^{NEO} mRNA-encoded protein is highlighted in turquoise. iBAQ, intensity-based absolute quantification. (Color version of figure is available online.)

multiple peptides derived from the mRNA-encoded protein were detected (Figure 5A), and quantification analysis demonstrated that the protein was among the top 30% most abundant quantified proteins (Figure 5B; also see supplementary Tables 2, 3) [23].

Discussion

The requirement to apply GMP standards during mRNA manufacturing depends on the intended use and whether the final advanced therapy medicinal product (ATMP) is investigational or authorized. According to the European guidelines concerning ATMPs, mRNA for transfection in DCs is considered a starting material [24]. GMP should be applied from the point when starting materials enter the production process, and this implies that in the authors' ATMP setting, the mRNA does not need to be produced under GMP conditions [25]. Nevertheless, QC testing (Table 2) on starting materials should be performed in accordance with GMP standards [18]. In the authors' facility, although not legally required, the mRNA production process is performed in a GMP-compliant manner to minimize quality variability and reduce the risk of batch failures, which cannot be afforded in this personalized setting, where time constraints are critical. Indeed, batch failures may increase manufacturing time by another 2–3 weeks.

Although quality requirements for gene vectors such as plasmid DNA and viral gene vectors are well defined in the Ph. Eur. [26], dedicated guidelines addressing QC of mRNA are lacking. Therefore, the authors had to make a risk-based selection of relevant quality attributes to be controlled to ensure the safety and biological activity of the mRNA. The specifications of the QCs were set based on experimental data, pharmacopoeia standards and the literature [26,27]. Based on the requirements for DNA, the authors decided to determine bacterial endotoxin content, sterility, concentration and purity. Specific quality assays were added to control degradation and the

presence of dysfunctional mRNA transcripts, as mRNA is very susceptible to RNases, which are ubiquitously present. Although measures are taken to remove potential RNases in the working environment during production, there is still a risk of RNase contamination and resulting mRNA degradation. In addition, residual incompletely linearized DNA plasmids can give rise to long, dysfunctional mRNA transcripts. As the integrity of mRNA is crucial to its potency, it is of critical importance to measure the size of the major mRNA transcript and assess the homogeneity of the mRNA transcripts (limited degradation and absence of long, aberrant transcripts), which is performed by CGE. The quality assessment approach described in this article was approved by the competent regulatory authority in Belgium (Federal Agency for Medicines and Health Products) as part of the investigational medicinal product dossier of MIDRIX^{NEO}, which is currently being evaluated in a phase 1 clinical study. In later clinical development, the quality assessment and specifications may have to be adapted and refined to obtain a more extensive characterization of the mRNA.

Ideally, a potency assay for mRNA in vaccines should assess the capability of the mRNA to give rise to immunogenic peptides. Immunogenicity of mRNA is a complex feature and depends on multiple factors, of which protein expression and HLA binding of the resulting peptides are major determinants [28]. Protein expression analysis of the TMG^{NEO} mRNA is performed by LC-MS/MS on the mo-DCs of the final MIDRIX^{NEO} vaccine. The use of this technology is generally applicable to patient-specific mRNA, as (i) it does not, in contrast to western blotting, require specific antibodies and (ii) only limited amounts of material are required (2×10^5 electroporated DCs). HLA binding of the neoantigens included in the mRNA is assessed by in silico prediction algorithms, which are predominantly based on deep neural networks, especially the commonly used NetMHC and NetMHCpan [29]. MS-based immunopeptidomics analysis is currently the only unbiased method for directly identifying the repertoire of tumor HLA-

binding peptides but is limited by its low sensitivity and requirement of large cell quantities [30]. Therefore, the authors are currently developing an approach for confirming HLA binding of the mRNA-encoded neoantigens in autologous cells through MS-based immunopeptidomics. Together with the protein expression analysis of the mo-DCs, this will provide the authors with a more comprehensive assessment of the immunogenicity of the TMG^{NEO} mRNA.

Conclusions

The authors present a small-scale and GMP-compliant production process of patient-tailored mRNA-encoding neoantigens. Validation runs showed that the process delivers mRNA of consistent high quality, as demonstrated by the full compliance of each run with the pre-defined release specifications and the low batch-to-batch variability. To the authors' knowledge, this is the first time a GMP-compliant mRNA production process and mRNA quality assessment have been described in detail. The production protocol and quality assessment can be adapted by other academic institutions that are likewise in early clinical development of mRNA-based neoantigen vaccines.

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Author Contributions

Conception and design of the study: JI, BV, LDC, KV, FI. Acquisition of data: JI, PD, KW, KH, SDS, RLM, NL, MB. Analysis and interpretation of data: JI, BV, KW, KV, FI. Drafting or revising the manuscript: JI, LDC, RLM, KW, MB, SDM, MP, LB, GG, SB, HJ, SVL, GL, TT, BM, KV, FI, BV. All authors have approved the final article.

Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2021.08.005.

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