CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Supplementary Material

Chromatographic purification with CIMmultus™ Oligo dT increases mRNA stability

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MATERIALS & METHODS

Chemicals

10X RNAPol Buffer (NEB, B9012S), RNAse inhibitor 40U/µL (NEB, M0314S), MgCl2 (1 M, Invitrogen, AM 9530G), ATP Solution, Tris buffered 100 mM (ThermoScientific, R1441), GTP Solution, Tris buffered 100 mM (ThermoScientific, R1461), CTP Solution, Tris buffered 100 mM (ThermoScientific, R1451), UTP Solution, Tris buffered 100 mM (ThermoScientific, R1471), ARCA 40 mM (NEB, S1411L), Pyrophosphatase, 100 U/ml (NEB, M2403L), T7 RNA polymerase 50U/µL (NEB, M0251L), Na2HPO4 (Sigma, 71636), Na2H2PO4 (Merck, 1.37018), EDTA (Kemika, 11368.08), NaCl (Honeywell, 31434), Na4P2O7·10H2O (Sigma, S6422) SLBX2665, HEPES (Merck, 1.10110) K52462110033, Tris (Sigma, T6066), NaOH (Honeywell, 30620), Glycine (Merck, 1.04201), Agarose (Sigma, A9539) and Boric acid (Sigma, B6768).

Methods

pDNA isolation

E. coli cell pellet (biomass) containing eGFP plasmid was kindly provided by BioMay AG (Vienna, Austria). Plasmid containing an eGFP construct with encoded 45 nt polyA-tail was purified using CIM™ HiP² Plasmid Process Pack 8 mL columns (BIA Separations, Ajdovščina, Slovenia) [18]. Briefly, biomass containing pDNA was resuspended in 50 mM Tris, 10 mM EDTA, pH 8.0. Alkaline lysis was performed at room temperature using 0.1 M NaOH and 0.5% sodium dodecyl sulfate. After 5 min lysis time, 3 M potassium acetate, pH 5.5 was added to a final concentration of about 1 M to neutralize the solution, followed by precipitation in 1 M $CaCl_2$. Coarse filtration was performed with

Sartopure PP3® filter (Sartorius AG, Göttingen, Germany), followed by a fine filtration through Sartopore® 0.80/0.45 µm filter (Sartorius AG, Göttingen, Germany). Conductivity of the $CaCl_2$-treated lysate was adjusted to 35 mS/cm with deionized water and the sample was loaded on CIMmultus™ DEAE 8 mL column (BIA Separations, Ajdovščina, Slovenia). After washing the column with 50 mM Tris, 10 mM EDTA, 0.6 M NaCl pH 7.2, the pDNA was eluted with 50 mM Tris, 10 mM EDTA,1 M NaCl pH 7.2. Elution fraction was buffer-exchanged into 50 mM Tris pH 7.2 using Vivaspin®-4 50K PES (Sartorius AG, Göttingen, Germany) column.

Plasmid linearization:

Plasmid DNA was incubated with NotI-HF (NEB) at 37°C for 60 min and inactivated with EDTA (15 mM final concentration). After inactivation linearized plasmid was purified by CIMmultus™ C4 HLD column. In brief, the linearization reaction mixture was adjusted to 2.5 M ammonium sulfate in 50 mM Tris, 10 mM EDTA, pH 7.2, then filtered through Sartopore® 2 0.45 µm filter (Sartorius AG) and loaded onto CIMmultus™ C4 HLD 8 mL column (BIA Separations, Ajdovščina, Slovenia). pDNA was eluted in a step gradient to 0.7 M ammonium sulfate. Eluate was buffer exchanged into 50 mM Tris pH 7.2 using Vivaspin®-4 3K PES (Sartorius AG, Göttingen, Germany) column.

IVT

All IVT reagents were preheated to 37°C. Preheated reagents listed in Table 1 were mixed in a 15mL plastic tube and incubated at 37°C. Reaction was monitored by CIMac PrimaS™ and quenched with EDTA when maximum mRNA concentration was reached. Quenched reaction mixture was divided equally into aliquots which were purified by different purification methods.

REAGENT:	FINAL Concentration:
Nuclease free water	
10x RNAPol Buffer (NEB, B9012S)	1x
RNAse inhibitor 40 U/µL (NEB, M0314S)	1 U/µL
$MgCl_2$ (Invitrogen, AM 9530G)	12 mM
NTP solution mix ThermoSci. R1441 (ATP), R1461 (GTP) R1451 (CTP), R1471 (UTP)	4 mM each
pIVTeGFP linearized plasmid	80 µg (20 ng/µL)
Pyrophosphatase, 100 units/mL (NEB, M2403L)	1 U/mL
T7 RNA polymerase 50 U/µL) (NEB, M0251L)	500 U/µg

mRNA purification

Purification by precipitation

Monarch® RNA Cleanup Kit (NEB, Ipswich, MA, USA) was used for extraction of mRNA from IVT according to manufacturer's instructions. Elution was measured by Nanodrop (UV absorbance at 260 nm) and diluted to 100 µg/mL with ddH_2O.

Purification by hybridization-affinity chromatography

Chromatographic purification was performed on AKTA Explorer 100 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) FPLC system composed of two pumps and a multiwavelength UV-Vis detector (2 mm flow cell path length). Unicorn software (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden) was used for instrument control and data acquisition. IVT mixture was diluted 1x in sample loading buffer (50 mM sodium phosphate, 2 mM EDTA, 1 M NaCl, pH 7.2) and loaded onto CIMmultus™ Oligo dT18 (C12) - 1 mL (2 µm channel size) column (BIA Separations, Ajdovščina, Slovenia) in mobile phase containing 50 mM sodium phosphate, 2 mM EDTA, 0.5 M NaCl, pH 7.2. After unbound IVT components eluted in flow-through and the UV 260 nm signal was stabilized, a wash step was performed with 50 mM sodium phosphate, 2 mM EDTA, pH 7.2, followed

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by step elution of polyadenylated mRNA with double deionized water.

ANALYTICS

Qualitative analysis by anion exchange chromatography

Experiments were performed on a PATfix™ HPLC system (BIA Separations, Ajdovščina, Slovenia) composed of quaternary pump, a multiwavelength UV-Vis detector (50 mm flow cell path length), a conductivity, and a pH monitor (BIA Separations). ClarityChrom® software (Knauer, Berlin, Germany) was used for instrument control and data acquisition and PATfix™ software (BIA Separations) was used for data analysis. CIMac PrimaS™ column with 0.1 mL bed volume and average channel size of 2 µm was used (BIA Separations, Ajdovščina, Slovenia). mRNA samples were equilibrated by a dilution with mobile phase A (MPA) (50 mM HEPES, pH 7.0). Injection volume was 25 µL. NTPs from the IVT reaction were separated in a linear gradient to 20% of mobile phase B (MPB) (50 mM HEPES, 200 mM Sodium pyrophosphate, pH 8.5). In the following isocratic elution pDNA is eluted and finally mRNA is eluted in a step to 50 % MPB, followed by another step to 100% MPB and then CIP with 0.1M NaOH + 1M NaCl (MPC) to regenerate the column. Finally, the column is equilibrated with (MPD) 1.5 M HEPES pH 7 and MPA.

Quantitative analysis by hybridization-affinity chromatography.

Experiments were performed on a PATfix™ HPLC system composed of quaternary pump, a multiwavelength UV-Vis detector (10 mm flow cell path length), a conductivity, and a pH monitor (BIA Separations). ClarityChrom software (Knauer, Berlin, Germany) was used for instrument control and data acquisition and PATfixTM software was used for data analysis. Affinity column CIMic Oligo dT18 (C12 Linker) was used with 0.1 mL bed volume and average channel size of 2 µm (BIA Separations). mRNA samples were equilibrated by a 10x dilution with Mobile phase A (MPA) (50 mM sodium phosphate, 2 mM EDTA, 0.5 M NaCl, pH 7.4). Injection volume was 200 µL. Samples were loaded in MPA (10 CV), then a wash step was performed with Mobile phase B (MPB) (50 mM sodium phosphate, 2 mM EDTA, pH 7.4, 35 CV) followed by elution with Mobile phase C (MPC) (double deionized water; 30 CV). Cleaning-in place (CIP) was performed with Mobile phase D (MPD) (0.5 M NaOH; 10 CV) before re-equilibration of the column.

Agarose gel electrophoresis

Prior to gel electrophoresis, samples were concentrated and buffer exchanged by centrifugal filtration. pDNA conformations were analyzed by agarose gel electrophoresis (Bio-Rad, Hercules, CA, USA) using 1 kb Plus DNA Ladder and SYBR Safe DNA gel stain both obtained from Invitrogen (Eugene, OR, USA). Agarose gel (0.8% density) was made by dissolving agarose (Sigma-Aldrich, St. Louis, MO, USA) in 1x TBE buffer (40 mM Tris, 20 mM boric acid, 1 mM EDTA pH 8.0). TBE was also used as the electrophoresis running buffer. 18 µL of pDNA samples and standards were applied to the gel. Electrophoresis was performed at 70 V for 100 min.

Capillary electrophoresis

The integrity of the mRNA samples was determined by capillary electrophoresis with a BioAnalyzer 2100 (Agilent, Santa Clara, CA, USA), using RNA 6000 Nano Kit (Agilent, Santa Clara, CA, USA), according to manufacturer's instructions.

SUPPLEMENTARY FIGURES

FIGURE S1

Agarose gel electrophoresis of mRNA heated at (1) 70°C for 5 min (2) not heated prior to electrophoresis.

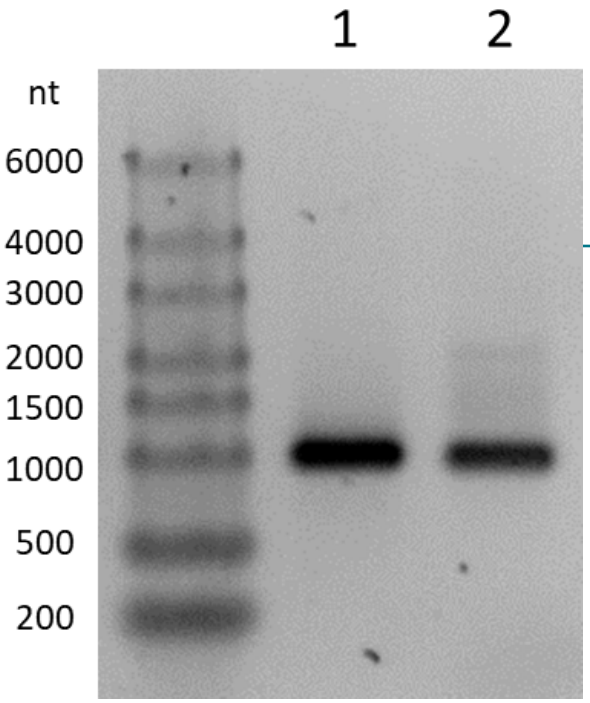

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FIGURE S2

BioAnalyzer electropherograms for mRNA prepared by A) dilution, B) Oligo dT and c) extraction kit at 37°C for 0-28 days.

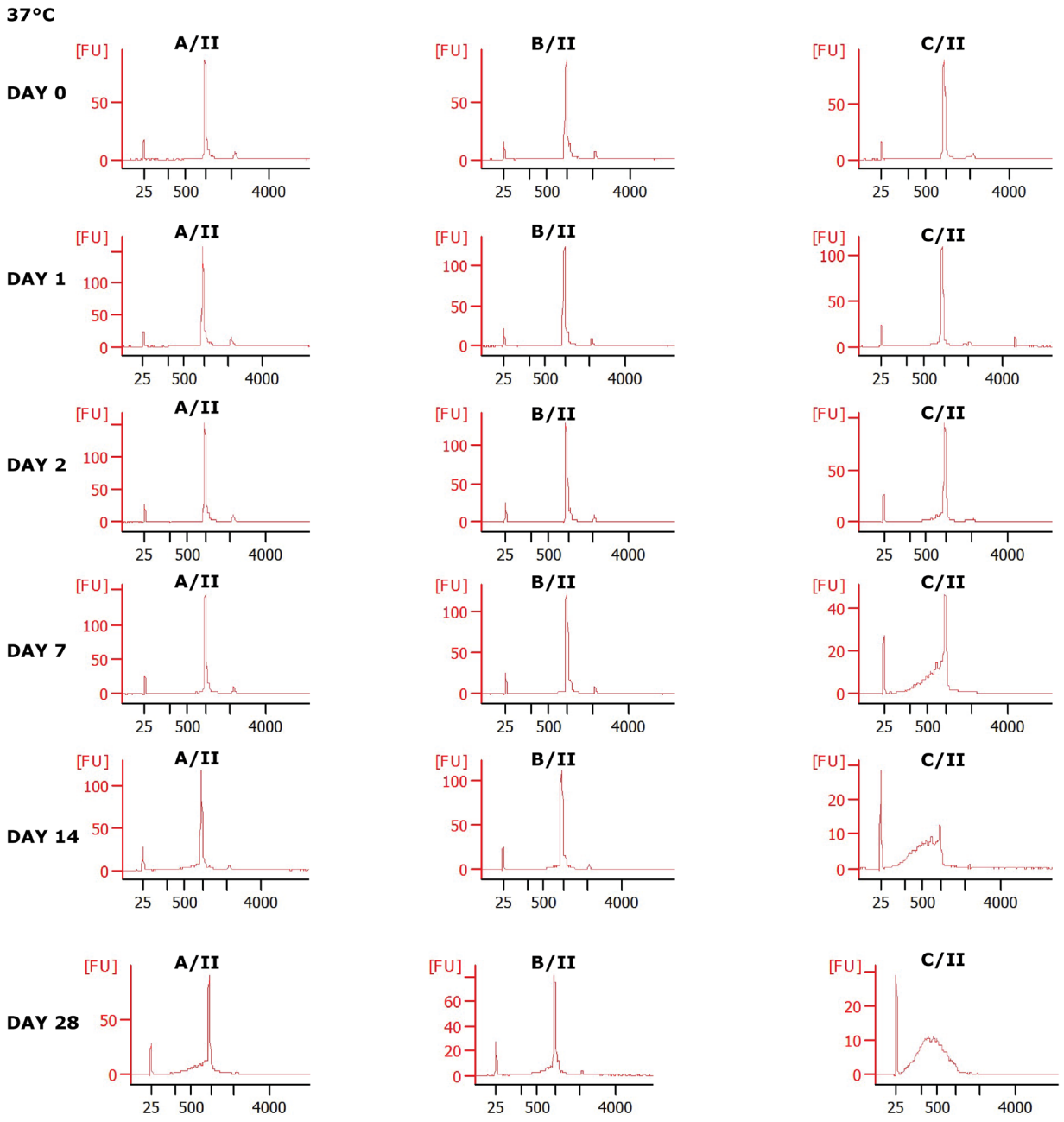
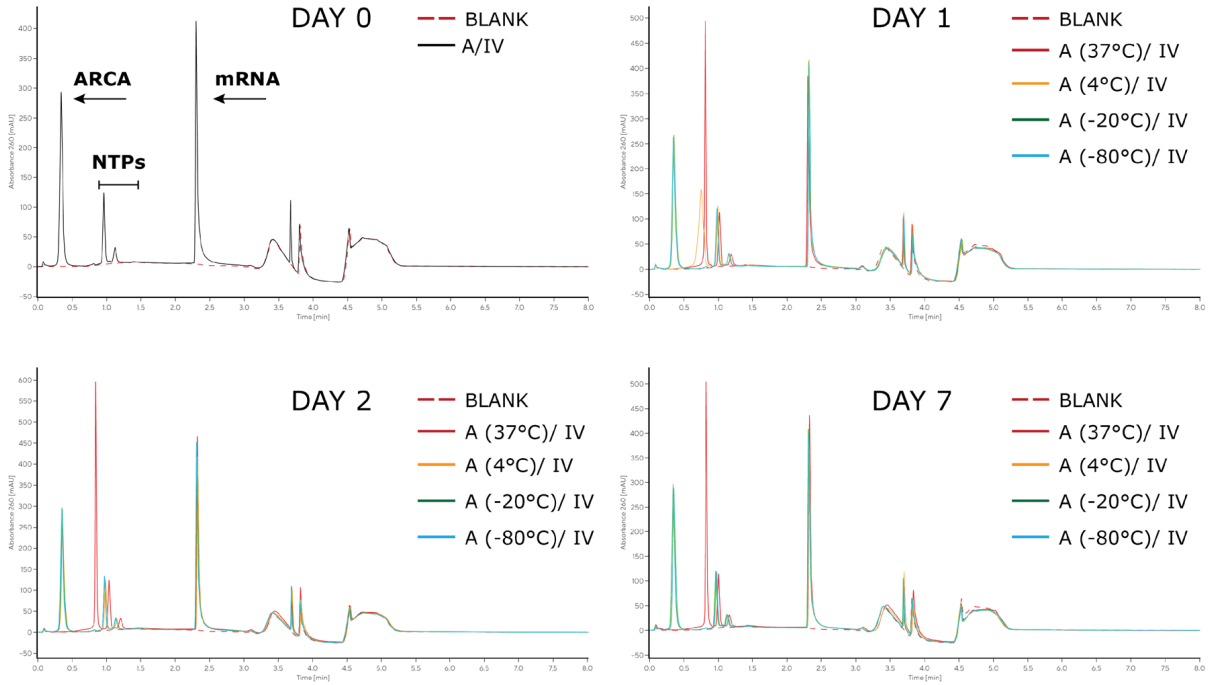


FIGURE S3

CIMac PrimaS analytics of diluted IVT mixture for up to 7 days.



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