Automation of mRNA synthesis and purification for effective template construct screening during mRNA vaccine development

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ABSTRACT

To simplify template screening and optimization during DNA construct development, we have automated the complete workflow on KingFisher Instruments in reaction volumes ranging from 100µL to 4mL, enabling synthesis and purification of mRNA in less than 5 hours. Template variants of target sequences in plasmid libraries, or completely synthetic DNA sequences already PCR amplified using a biotinylated forward primer, serves as the starting point for the automated workflow. On the KingFisher platform, the PCR-product is purified by immobilization to streptavidin coated magnetic beads and the bead-DNA complex is used directly as template for in vitro transcription. The immobilized template is reusable for in vitro transcription at least six times, enabling synthesis of gram scale mRNA from nanograms of plasmid preparations.

As an example, starting from 10ng plasmid DNA you get enough PCR-product to synthesize 10mg of mRNA without template reuse. By KingFisher automation, it is possible to synthesize and purify mRNA from 96 different DNA constructs in 5mg scale, within one working day. With 6 times reuse of the most promising templates, the original 10ng plasmid input, enables production of more than 30mg of mRNA for further studies.

Here we present data from automated solid-phase in vitro transcription followed by generic capture purification of the synthesized RNA, including yield, purity, and integrity.

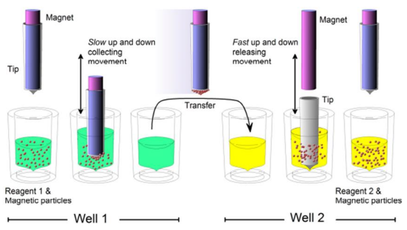
INTRODUCTION

During the discovery phase of a new mRNA vaccine development, there are many different elements to consider, both when it comes to base compositions, length and modifications, UTRs, capping, polyA-tail in addition to finding the optimal reaction conditions. This screening process can be significantly simplified, by assembling a reusable, PCR-amplified template DNA and performing solid-phase in vitro transcription, by immobilizing the template on magnetic beads. Purification of the synthesized mRNA is also easily performed on magnetic beads.
The screening of up to 96 constructs in parallel, is easily automated on the KingFisher instruments. Scale up of in vitro transcription and purification of the most promising constructs, is performed on the same instrument in a 24 well format, which can handle from 200µL up to 5 mL. With template reuse, this enables the synthesis of more than 100 mg of mRNA in each well.

MATERIALS

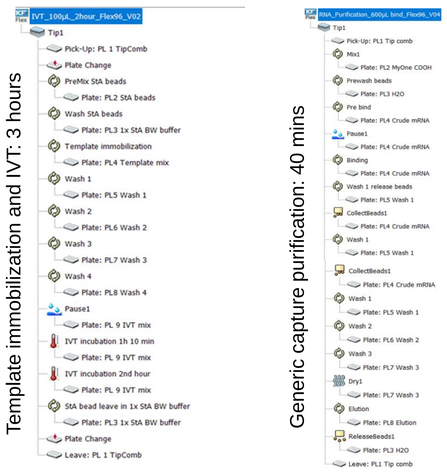
Dynabeads™ Streptavidin for In Vitro Transcription (Thermo Fisher SKU 49010D)
Dynabeads™ Carboxylic Acid for RNA Purification (Thermo Fisher SKU 49020D)
Dynabeads™ 1.5 x RNA Binding buffer (Thermo Fisher SKU 37035D)
Platinum™ SuperFi II PCR Master Mix (x100) (Thermo Fisher SKU 12368010)
SequalPrep™ Long PCR Kit with dNTPs (Thermo Fisher SKU A10498)
MEGAscript™ T7 Transcription Kit (x25 rxn) (Thermo Fisher AM1333)
mMESSAGE mMACHINE™ T7 Transcription Kit (x25) (Thermo Fisher AM1344)
Qubit™ 1X dsDNA High Sensitivity (Thermo Fisher SKU Q33230)
Qubit™ RNA BR assay kit (Thermo Fisher SKU Q10211)
Micro BCA Protein Assay kit (Thermo Fisher SKU 23235)

KingFisher Flex/Apex principle



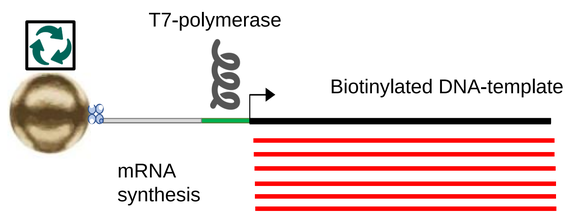
Inverse magnetic bead processing. Magnetic rods covered with tips are slowly moved up and down in bead suspension to collect the beads (well 1). Beads are transferred to the next plate where the beads are released in the reagent by tip mixing in the solution (well 2).

KingFisher Flex/Apex Script layout



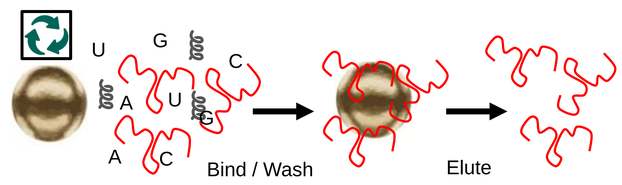
METHODS

Solid-Phase In Vitro Transcription



Dynabeads Streptavidin for In Vitro Transcription:
The target sequence is PCR-amplified using a biotinylated forward primer. The PCR product is purified by immobilizing it to Dynabeads™ Streptavidin for In Vitro Transcription. The bead-DNA complex is used directly as template in IVT. Following IVT, the template is removed from the synthesized mRNA by magnetic separation and can be recycled at least 6 times in consecutive IVT reactions (data not shown).

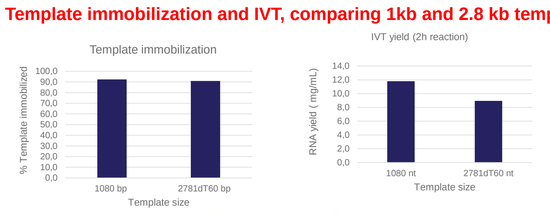
Generic Capture RNA Purification



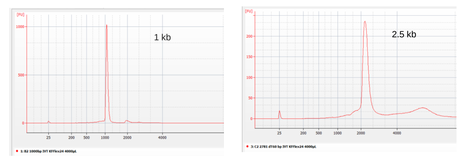
Dynabeads Carboxylic Acid for RNA puriification :
After magnetic removal of the DNA template, remaining IVT reagents are removed by generic capture of the mRNA onto carboxylic acid activated Dynabeads in a binding solution. It is a simple bind, wash elute reaction. The beads can be reused at least 6 times (data not shown)

Scale up to 4 mL on KingFisher Flex 24

Template immobilization and IVT, comparing 1kb and 2.8 kb templates

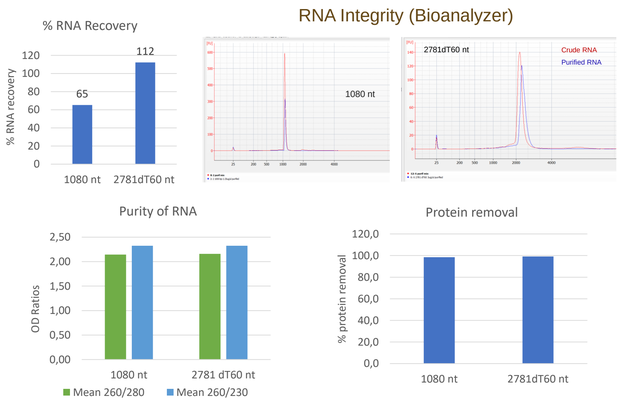


RNA Integrity (BioAnalyzer)



Both 1kb and 2.8kb templates are efficiently immobilized and give high RNA yields in IVT, in 4 mL reaction volume, producing more than 30mg of RNA in each well, without reuse of the template.

RNA purification, comparing 1kb and 2.5 kb RNA transcripts



Purification of crude IVT RNA in 4 mL volumes on KingFisher Flex 24, gives the same yield, integrity and purity as in lower scale.

Sequence comparison of RNA transcribed from plasmid template and PCR generated templates

Seq #	Sample type sequenced	Max Scr	Tot Score	Query cover	E val	Per. Ident	Acc. Len	Accession Query
1	Linearized plasmid DNA(1) (5330 bp)	Reference sequence					2798	
2	PCR-product(2) SeqPrep (2798 bp)	5053	5053	97%	0.0	**100.00%**	2736	Query_20806
3	PCR-product(3)-SuperFi (2798 bp)	5053	5053	97%	0.0	**100.00%**	2736	Query_20807
4	IVT-RNA from linearized plasmid DNA	4708	4708	91%	0.0	**99.96%**	2552	Query_20808
5	IVT-RNA from linearized plasmid DNA	4715	4715	91%	0.0	**100.00%**	2553	Query_20809
6	IVT-RNA from SeqPrep PCR prod – 1st use	4715	4715	91%	0.0	**100.00%**	2553	Query_20810
7	IVT-RNA from SuperFi PCR prod – 1st use	4715	4715	91%	0.0	**100.00%**	2553	Query_20811
8	IVT-RNA from SeqPrep PCR prod – 3rd reuse	4715	4715	91%	0.0	**100.00%**	2553	Query_20812
9	IVT-RNA from SuperFi PCR prod – 3rd reuse	4715	4715	91%	0.0	**100.00%**	2553	Query_20813
10	IVT-RNA from SeqPrep PCR prod – 6th reuse	4711	4711	91%	0.0	**99.96%**	2553	Query_20814
11	IVT-RNA from SuperFi PCR prod – 6th reuse	4715	4715	91%	0.0	**100.00%**	2553	Query_20815

Pairwise alignment

```
Sequences (1:2) Aligned. Score: 100
Sequences (1:3) Aligned. Score: 100
Sequences (1:4) Aligned. Score: 99.8041
Sequences (1:5) Aligned. Score: 100
Sequences (1:6) Aligned. Score: 100
Sequences (1:7) Aligned. Score: 100
Sequences (1:8) Aligned. Score: 100
Sequences (1:9) Aligned. Score: 100
Sequences (1:10) Aligned. Score: 99.9608
Sequences (1:11) Aligned. Score: 100
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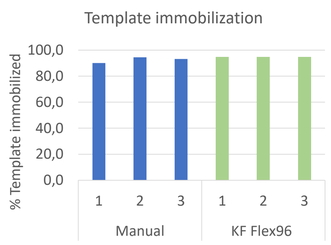
Sanger sequencing Showed 100% identity between mRNA synthesized from PCR amplified template and linearized plasmid template

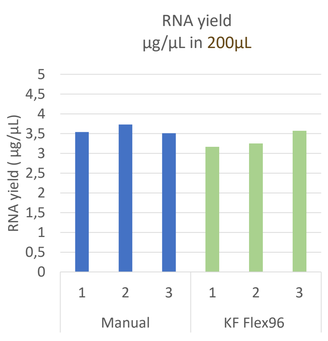
RESULTS

RNA Synthesis on KingFisher Flex 96

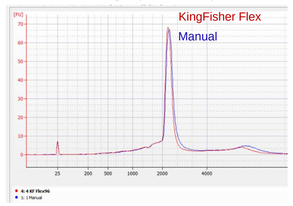
Manual vs automated performance



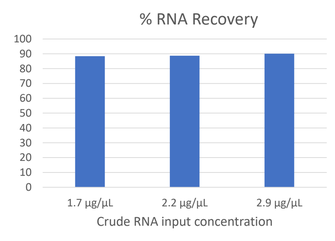
Manual and automated protocols perform equally well. Figures show template immobilization efficiency, RNA yield and RNA integrity from a 200µL reaction, using 2µg of PCR-amplified template immobilized to 2mg of Dynabeads Streptavdin for in vitro transcription, using MEGAscript reagents for 1 hour.
Template immobilization was more than 90%, IVT yield around 3.5 mg/mL and integrity was the same as shown by Bioanalyzer electropherogram overlays.

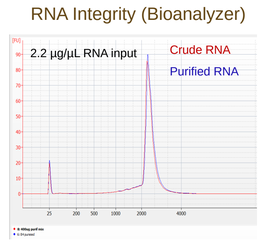


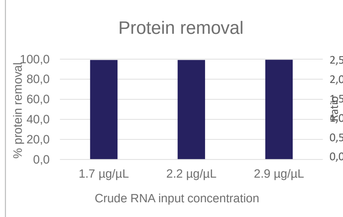
RNA Integrity (Bioanalyzer)

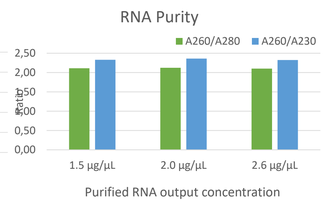


RNA Purification on KingFisher Flex 96







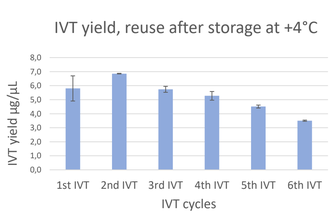


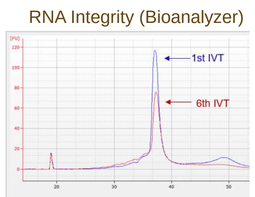
The purification step on KingFisher Flex using 200µL input RNA in a 600µL binding volume, shows a recovery close to 90%, with different RNA input concentrations.
Purity assessed by Nanodrop is acceptable, and a Mico BCA assay shows more than 99% protein removal. Integrity is shown by the BioAnalyzer electropherogram overlays.

Storage Stability of Template on Beads

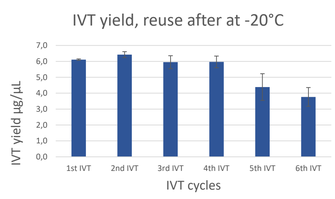
A 2.8 kb Biotinylated template was immobilized to Dynabeads Streptavidin, and reused every 3rd day, in IVT on KingFisher APEX96, after storage at different temperatures. IVT volume was 200µL volume and reaction time was 2 hours using MEGAscript.

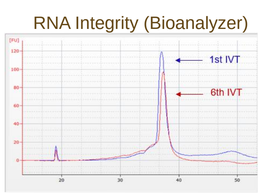
Templates can be stored on beads at 4ºC for reuse in IVT





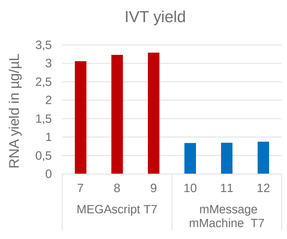
Templates can be stored on beads at -20ºC, for reuse in IVT

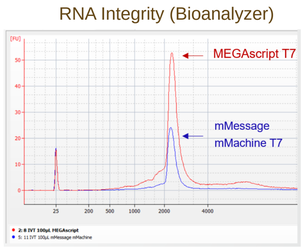




Freezing and thawing of beads with immobilized template 6 times, still give considerable yield of in vitro transcript, showing the flexibility and robustness of the technology.

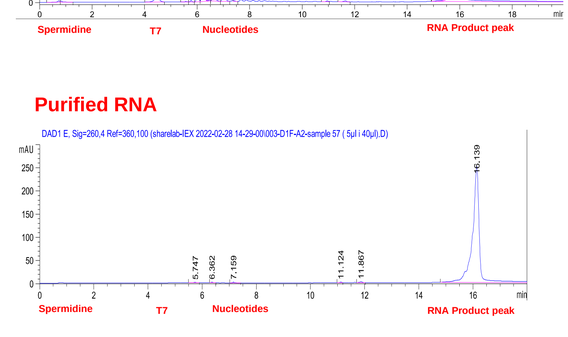
Screening of IVT-reagents





Screening of IVT-reaction conditions and reagent mixes can be done in parallel on KingFisher instruments, here showing different RNA yield comparing MEGAscript and mMessage mMachine. 100µL IVT for 2-hours was compared using a 2.8kb template immobilized to beads. The reduced IVT yield for mMessage mMachine reagents is caused by the lower NTP concentration and in-process capping. This screening was performed on KingFisher Flex 96.

Binding Capacity for Purification



Different amounts of the synthesized 2.5kb crude IVT RNA was purified on 600µg of Dynabeads Carboxylic Acid beads, with high recovery (more than 80%), showing that the beads can efficiently purify RNA in amounts ranging from at least 0.1 to 1 mg of RNA per mg of beads. The purification was performed on KingFisher Flex using 600µg beads in 600µL binding volume, with RNA input from 94µg up to 720µg.

Purity of mRNA after purification

IEX HPLC comparing Crude & purified RNA

Crude IVT-RNA

Purified RNA

Generic capture purification removes remaining reagents from the IVT-reaction, like NTPs, enzymes and spermidine. Micro BCA assay shows that more than 99% of the protein is removed.
DNA template released from the beads during IVT compose less than 0.01% of the RNA in the crude IVT, without any DNase treatment, as quantified by qPCR and LC-MS/MS (data not shown).

CONCLUSIONS

- Automated workflow enables high throughput screening
- Template immobilization, IVT and mRNA purification of 96 samples performed in less than 5 hours
- Bead platform optimized for handling and safety purposes.
- Simple, flexible and highly scalable workflow
- Minimized need for plasmid preparation
- Template reuse enables low manufacturing footprint
- Same technology from research and development to vaccine manufacturing
- Beads compliant with GMP ancillary material
- Regulatory support documentation available

ACKNOWLEDGEMENTS

Manolito Torralba, for help with the sequencing project