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Optimisation of capillary gel electrophoresis method for enhanced separation of mRNA shortmers

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Abstract

Optimisation of capillary gel electrophoresis method for enhanced separation of mRNA shortmers

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Advancements in the field of modified messenger RNA (mRNA) has led to new ventures in the pharmaceutical industry. However, new drug products demand new analytical methods to ensure the efficacy and purity of the drug. Capillary gel electrophoresis (CGE) with UV detection shows great potential for separation of mRNA samples due to the equal mass-to-charge ratio of mRNA and the flexible parameters of the CGE methods.
This thesis investigates the optimal parameters of the capillary electrophoresis method, sample treatment procedure and sieving medium composition for enhanced shortmers separation of mRNA by CGE analysis. An RNA ladder with 100-1000 nucleotides and EPO mRNA with 900 nucleotides were used as model compounds. The effect of capillary dimensions and separation temperature on the resolution of the RNA peaks was established through comparative experiments. Sample treatment processes were evaluated to achieve an optimal conformation of the mRNA for CGE analysis. By heating the mRNA sample for 15 min at 80°C all multimers were seemingly eradicated. Moreover, it was found that addition of 4 M of urea to mRNA sample before heating resulted in improved peak shape. A sieving medium consisting of a mix of the two polymers polyvinylpyrrolidine (PVP) and hydroxyethyl cellulose (HEC) proved to have beneficial qualities for separation. The addition of sucrose as viscosity modifier in the sieving medium surprisingly further enhanced the resolution. Moreover, during the project a heavy wash was established which drastically improved repeatability of the analyses through more efficient regeneration of the capillary.

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Populärvetenskaplig sammanfattning

Nya framgångar kring modifierat messenger RNA (mRNA) har möjliggjort utveckling av en ny kategori av läkemedel med potential att kunna övervinna de hinder som man tidigare påträffat inom vissa sjukdomsområden. Återhämtning av hjärtvävnad efter hjärtinfarkter är ett exempel på område där modifierat mRNA tros ha stor potential och som nu utforskas av läkemedelsföretag. Fördelar med mRNA som läkemedel jämfört med tidigare beprövade metoder är bland annat möjligheten att kunna producera vilket typ av protein som helst med kroppens eget maskineri. Detta helt utan risk för bestående men orsakat av mutagenes.

De strikta kvalitetskrav som läkemedelsbranschen ständigt jobbar under kräver effektiva och omfattande analysmetoder anpassade för varje aktiv läkemedelsingrediens. Med dessa metoder vill man bland annat kunna visa på hållbarheten av provet under förvaring och hantering. Detta för att kunna försäkra att det som når patienterna är säkert och av hög kvalitet. Kapillärgelelektrofores är en analysmetod som visat sig lämplig för nukleinsyraprover. Med dessa provers enhetliga massa/laddning-förhållande sker separationen med avseende endast på storlek och separationsförhållandena kan upprepas eller anpassas tack vare det flexibla separationsmediet. Denna metodflexibilitet gör det också komplext att hitta den optimala metoden för varje enskild analys då flera parametrar kan varieras och kommer också ha en inverkan på varandra. Vid goda separationsförhållanden anpassade för det specifika syftet och provet kan dock en fullständig separation erhållas med enbart ett par mikroliter prov som åtgång.

Syftet med detta projekt var att optimera den redan befintliga kapillärgelelektroforesmetod som används för separationsanalys av mRNA prov. Målet var att öka upplösningen hos separationen av de mindre fragment som förekommer innan den förväntade huvudtoppen. För att nå detta mål undersöktes alternativa parametrar på kapillärelektroforesinstrumentet, komposition av separationsmediet och förbehandling av analysprovet både genom litteratursökning och experimentellt arbete.

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List of abbreviations

CE	Capillary electrophoresis
CGE	Capillary gel electrophoresis
Da	Dalton
DNA	Deoxyribonucleic acid
EOF	Electroosmotic flow
EPO	Erythropoietin
FACE	Fragment Analyzer Capillary Electrophoresis
HEC	Hydroxyethyl cellulose
ID	Inner diameter
IHD	Ischaemic heart disease
LPA	Linear polyacrylamide
mRNA	Messenger RNA
nt	Nucleotides
PP	Polypropylene
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
RNase	Ribonuclease
VEGF	Vascular endothelial growth factor

1 Introduction

According to a vast international statistical study the globally leading cause of premature death during the 21st century is ischaemic heart disease (IHD) (Wang, Naghavi et al. 2016). As the world population is growing older with improved living conditions the lethal medical conditions of poor diets and exercising habits grow increasingly common. Though treatment of IHD is possible it has thus far mostly been oriented towards relieving symptoms instead of purely curative, leaving the infarct affected region of the heart with scar tissue and faulty function. Vascular endothelial growth factor A (VEGF-A) has the ability of inducing the growth of heart muscle tissue which otherwise holds a very low regenerative ability (Hadas, Katz et al. 2017). However, a difficulty of VEGF-A has been the delivery method to the relevant site for sufficient response. In a recent project AstraZeneca teamed up with the RNA specific company Moderna, to develop a novel drug using modified messenger RNA (mRNA) to deliver the growth factor protein and thereby achieve a sufficient though transient regrowth of heart tissue (Mullard 2016). So far, the modified mRNA has shown potential as a novel drug modality. However, new active pharmaceutical ingredients and drug components call for extensive investigation before being released into further development stages.

With the pharmaceutical industry being an ever-evolving business where new solutions are in constant request to help cure people from diseases and genetic disorders, analytical method development for quality testing is a significant part of the process to determine the suitability of a drug. The strict regulations on pharmaceutical development make it essential for the industry to have efficient and reliable methods to establish the stability and purity of each new drug. So, along with the discovery of a potential new kind of active pharmaceutical ingredients come a lot of method development and optimization, all to ensure the safety and wellbeing of the patients. Finding functional orthogonal methods for the build-up of quality testing to ensure the efficacy and safety of the drug is essential (Olsen, Sreedhara et al. 2017).

Capillary Gel Electrophoresis (CGE) has been noted for its compatibility with nucleic acid as for the equal mass-to-charge ratio of the molecules and the very small amount of sample necessary for extensive analysis. Additionally, the flexibility of the sieving medium allows for efficient separation of various molecule lengths and analysis purposes by adjustment of polymer concentration and other components (Petersen and Mohammad 2001).

1.1 Aim

The aim of this project was to develop an improved CGE method with sieving medium for enhanced separation of shortmers from the mRNA main peak. To achieve this, the method optimisation focus was mainly on three different areas; instrument parameters, sample treatment procedure and sieving medium composition.

Since the modified mRNA drug products are still in early development phase the optimisation experiments were carried out using EPO mRNA which also contains modified nucleotides

and is of similar length as typical mRNA, and RNA ladder 0.1-1 kb where the fragment sizes are defined and the resolution of separation can be easily visualised and evaluated.

2 Theory

2.1 mRNA as therapeutic agent

Great advancements have been made in the field of RNA in recent years, leading to new visions of its capacities in the medical drug industry (Kallen and Theß 2014). mRNA is the middle step between DNA and the expression of proteins in cells. It acts as a blueprint to all proteins that are assembled in our body, making synthetically engineered mRNA a good prospect for generating proteins of interest in a wide variety of disease areas (Van Lint, Heirman et al. 2013). Already in 1992 a study was carried out on rats where mRNA from healthy rats was injected into diabetic rats and an increase in the production of a specific peptide previously lacking in these rats could be confirmed (Jirikowski, Sanna et al. 1992). However, despite this success in the pioneering field, the science of the following decades would mostly come to focus on the prospects of DNA-based therapeutics as it was regarded as a more stable molecule (Sahin, Kariko et al. 2014).

One of the major difficulties originally encountered in laboratory handling of the mRNA molecule is that it is easily degraded in an ordinary environment due to the abundance of ribonuclease (RNase) (Kallen and Theß 2014). These enzymes are present in almost all eukaryotic and prokaryotic cell types and play an important role in the nucleic acid metabolism and also serve as a defence against invading microorganisms. To prevent these enzymes from degrading the RNA of interest precautions have to be made regarding the lab environment. Gloves and lab coats must be worn at all times when handling the samples and special areas and equipment in the lab should be dedicated solely for RNase free purposes (Ambion 2012, Sahin, Kariko et al. 2014). Other than the risk of degradation by RNase, stability of the RNA molecule is not a major issue. It has recently been reported that in an RNase-free environment it can be kept at room temperature for more than 2 years without being significantly degraded (Sahin, Kariko et al. 2014).

Another challenge that must be dealt with when pursuing mRNA as a possible drug agent is to prevent the activation of the immune system. This has been achieved by replacing the uridine nucleosides, which are known to activate several receptors of the immune system, with pseudouridine and thereby not only evading the immune system but also increasing the efficiency of translation (Weissman and Karikó 2015). Several other important structural compositions of the mRNA are also looked at to achieve optimal stability and translational efficiency. These include the cap structure, the 5’ untranslated region, the open reading frame, the 3’ untranslated region and the polyA tail which are all important factors for the overall efficacy of a potential drug (Yamamoto, Kormann et al. 2009).

Compared to targeting DNA as a way of controlling protein expression and functions, mRNA has great advantages. The mRNA does not need to be delivered into the nucleus of the cell. It is instead active in the cytoplasm, thereby avoiding the tough delivery through the nuclear membrane (Yamamoto, Kormann et al. 2009). Furthermore, mRNA does not interact with our genes and will therefore not comprise any risk of mutagenesis. It is also a transient molecule and will not produce the resulting protein forever (Van Lint, Heirman et al. 2013).

Though the stability of modified mRNA has been significantly enhanced with the latest breakthroughs in the field, it is still an issue to consider as mRNA is explored as therapeutic agent for new drug development. The process of development, manufacturing and storage subjects the drug product to risks of degradation by exposure to heat, hydrolysis, oxidation, ribonucleases etc. The degradation results in shorter fragments of mRNA i.e. shortmers. This makes it essential to develop thorough and efficient methods for stability and purity control of the prospective drug products.

2.2 Capillary electrophoresis

Capillary electrophoresis (CE) is a very suitable method for the separation of biomolecules and can also be an orthogonal method to liquid chromatography, a well-established separation tool in many labs. CE includes a set of different modes with the common property of using µm thin capillaries and an electric field in the separation process (schematic CE instrument set up in *figure 1*) (Khaledi 1998). There are several factors that affect the separation in CE. Capillary parameters, temperature, sieving medium composition, pH and sample treatment and composition are some of the essential factors that should be carefully considered for each unique analysis (Petersen and Mohammad 2001).

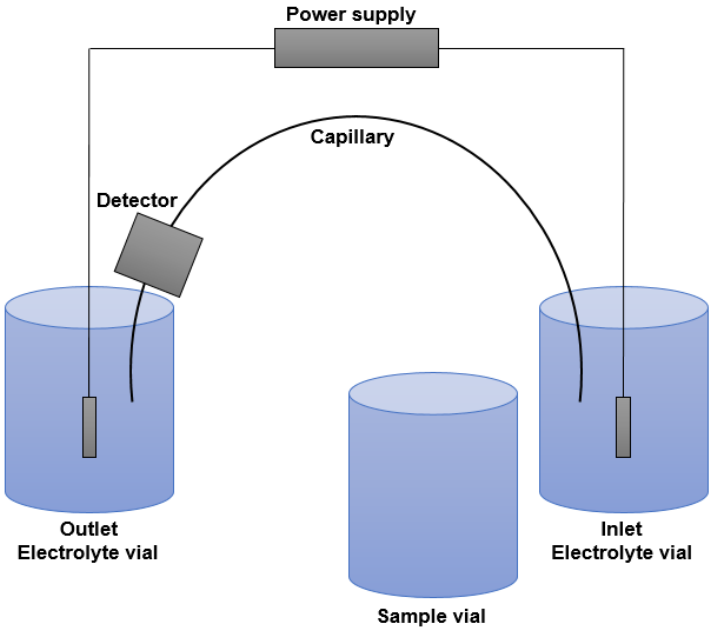

Figure 1. A schematic picture of the capillary gel electrophoresis instrument set up.

The usage of capillaries gives advantages in several aspects over the precursor slab gel electrophoresis including the minute amount, in the range of nanoliters (nl) of sample needed for each run. The injection volume for a capillary electrophoresis run can be determined by the Poiseuille equation (*equation 1*) (Petersen and Mohammad 2001):

$$\mathbf{V} = \frac{\Delta \mathbf{P} \mathbf{d}^{4} \boldsymbol{\pi} \mathbf{t}}{\mathbf{128} \boldsymbol{\eta} \mathbf{L}} \qquad \textit{Equation 1}$$

where ΔP is the pressure difference between the capillary ends (Pascal), d the inner diameter of the capillary (m), t the amount of time the pressure is applied (s), η the viscosity of the fluid (Pascal-seconds) and L the total length of the capillary (m).

2.2.1 Capillary gel electrophoresis

The principle of separation with Capillary Gel Electrophoresis (CGE) is the same as for conventional slab-electrophoresis; using an electrical field the molecules move through a gel (or sieving medium), where the separation is driven by the charge of the molecules and different capacities to move through the medium. For molecules with similar mass-to-charge ratio this results in a separation by size as bigger molecules are more hindered than smaller molecules when moving through the sieving medium and will therefore migrate slower and elute later. CGE is a well suited method for the separation of nucleic acids as these molecules vary in length but have a fairly constant charge-to-mass ratio and the separation will therefore solely be dependent on size (Petersen and Mohammad 2001).

The Ogston theory can be used to describe the movement of the short strands of nucleic acids through the entangled polymer solution that constitutes the sieving medium. The theory states that when the biomolecule radius is smaller or in the same size range as the pores of the polymer entanglement the molecules behave as rigid spheres and the separation, under an electric field, is based on the different ability of the molecules to move through the pores (Durney, Crihfield et al. 2015). However, this theory cannot fully describe the mechanism of movement and separation of nucleic acids in CGE as these molecules do not have a rigid spherical form but are in fact rod like with capabilities of deforming and unfolding. Additionally, the entangled polymers provide some flexibility in their network and thus the pore size of the medium may change during the separation (Todorov and Morris 2002). The reptation migration model instead describes the capability of longer nucleic acids to move "head first" through the polymer pores (Khaledi 1998). However, as the peak resolution decreases it might instead be worthwhile to consider separation in a polymer solution of less entanglement for larger nucleic acid fragments (Durney, Crihfield et al. 2015).

Especially for CGE it is important to maintain proper control of the pH of both the sample and sieving medium. A change in pH could affect the charge of the sample and thereby its ability to move through the capillary, it could also have a grave impact on the suppression of the electroosmotic flow (EOF) which would cause poor and irreproducible separation results.

This could indirectly also have an effect on the current and thereby also the heat distribution in the system (Petersen and Mohammad 2001).

Some of the advantages of CGE instead of the simpler versions of electrophoresis are that the thin capillaries give a very effective heat dissipation which in turn enables a stronger electric field and thereby shorter run times and less band broadening. It also has high sensitivity and very small amounts of samples are needed for analysis (Heller 1997). Additionally, a UV-detector can be directly coupled to the instrument, thereby eliminating any further preparation of the sample for detection. Moreover, the separation media composition is flexible and can be optimized for different types of samples and the analysis is easily automated (Todorov and Morris 2002) (Heller 2001).

2.2.2 Electroosmotic flow

The inner wall of untreated capillaries consists of fused silica. The properties of the inner walls can be modified. When using NaOH solutions in the initial washing of a fused silica capillary the original composition is fully hydrolysed to several types of weakly acidic silanol (SiOH) groups (Khaledi 1998, Petersen and Mohammad 2001) which are ionized in any solution above pH 2 (Heller 1997). This charged area then attracts free ions of opposite charge generating a thin layer, a Debye layer, of mobile ions. As an electric field is applied over the capillary, parts of the loosely held layer will start to migrate and through viscosity also influence movement of the neighbouring layers of fluid. This phenomenon is called electroosmotic flow (EOF) (Ghosal 2004). Although the movement induced by EOF is an important asset in some modes of CE, such as capillary zone electrophoresis, it needs to be suppressed for functional separation of DNA and similar biomolecules in CGE. Polymer coating by either covalent bonding or dynamic coating is regularly used to regulate the EOF. Kaneta *et al.* (Kaneta, Ueda et al. 2006) focused especially on polyvinylpyrrolidone (PVP) solutions for dynamic coating to the EOF suppression. They found that the relatively low viscosity of these solutions, as well as the ability to adsorb to the inner capillary wall, made PVP efficient for dynamic coating. Dynamic coating has proven to be advantageous in some cases compared to the more permanent covalently bound suppressors that often need complex procedures of preparation, which causes problems with reproducibility (Znaleziona, Petr et al. 2008).

2.2.3 Resolution

There are several parameters in CGE that affect the performance of the separation. One of the key parameters is the resolution of the peaks. Regarding the sieving medium a general idea for good resolution is that they should be highly viscous/entangled (Sartori, Barbier et al. 2003). Although this may increase the sieving mechanism of the medium it makes the process of filling and depleting the capillary significantly more difficult (Boulos, Cabrices et al. 2008). The content of the sample should also be taken into consideration for the sieving medium composition, especially the size of the RNA fragments as the molecular weight of the polymer will influence the pore size (Sartori, Barbier et al. 2003). The capillary length and

heat distribution capacity, which are discussed further in section *"2.2.4 Capillaries"* are other factors with great influence on the final resolution.

The resolution (R) between two peaks can be calculated using *equation 2* (Boulos, Cabrices et al. 2008):

$$\mathbf{R = \sqrt{2\ln(2)}\,\frac{t_2 - t_1}{w_1 + w_2}} \qquad \textit{Equation 2}$$

where t_1 and t_2 is the migration time of two subsequent peaks and w_1 and w_2 their respective half-height peak widths. In the work of De Scheerder *et al.* (De Scheerder, Sparén et al. submitted) it was found that the half-height width of some peaks was variable, therefore *equation 3* was used to calculate the resolution.

$$\mathbf{R = \frac{\Delta t}{\Delta F}} \qquad \textit{Equation 3}$$

where Δt is the difference in migration time between the two peaks and ΔF is the size difference in nucleotides divided by 100.

Conformation of the molecules in the sample also has an effect on the resolution of the separation. By eliminating alternative conformations such as transient folds, secondary and tertiary structures and dimers etc. band width can be decreased and thereby a better separation is obtained (Todorov, de Carmejane et al. 2001). This has previously been attempted by addition of chemicals, such as urea, directly to the sample vial or sieving medium (Harris, Baulcombe et al. 2017) (Lambert and Draper 2012) or by heating the sample and then cooling it again (De Scheerder, Sparén et al. submitted) (Skeidsvoll and Ueland 1996).

2.2.4 Capillaries

The most common capillaries used in CGE today are made of fused silica and have an inner diameter (ID) between 25-100 µm, making them very fragile. To improve ease of handling the capillaries are also coated with an outer layer of polyimide, which makes them more flexible and sturdier. However, a detection window must be left uncoated to enable on-capillary detection, e.g. by UV. The composition of the inner surface of the capillary is very important as it often is in direct contact with both the sieving medium and analyte. It is also here the potential EOF will arise, as described in *"2.2.2 Electroosmotic flow"*.

It is also possible to use permanently coated capillaries, with chemicals covalently bound to the inner wall, to aid the suppression of EOF and adsorption of analytes to the capillary walls etc. However, these are often more expensive due to the extensive manufacturing process and run the risk of deterioration after several runs, reducing the reproducibility of the method (Petersen and Mohammad 2001). Additionally, they often lack an extended detection window for improved sensitivity of detection.

Regeneration of the capillary is another important process for a reliable and robust CGE analysis. The ability to create the same conditions for all runs are essential for good reproducibility. This includes both a complete cleaning of the capillary after analysis to get rid of any buffer and sample residues as well as repeatedly creating the same inner surface for each analysis. Another essential part for enhancing reproducibility is to frequently change the electrolyte vials, as the concentration of ions will change over time as they migrate back and forth in the capillary under the influence of the applied electric field (Petersen and Mohammad 2001).

The choice of dimensions of the capillary used also has an effect on the quality of the analysis. A longer capillary enables a better separation although at the same time the possibility to maintain a higher V/cm is reduced as the system meets its limits of applied voltage. In turn, a wider ID allows for more sample to be injected, thereby increasing the detection levels. However, a wider capillary also lowers the heat transfer capacity and Joule heating may become a problem resulting in band broadening, thus lowering the resolution of the separation. It is therefore always important to consider these trade off parameters for the specific run (Khaledi 1998).

2.3 Sieving medium

In contrast to what the name implies, CGE is most often not carried out with a cast gel as separation medium. Although early usage of CGE did mimic the procedures of slab-electrophoresis and used agarose or other solid gels as separation matrix in the capillaries this soon proved to be inefficient. Gel breakdown, bubble formation and severely limited lifetime of the capillaries were common issues when using crosslinked polyacrylamide gels (Heller 1997) (Heller 2001). Polymer solutions are in contrast, today used to separate nucleic acids in CGE. These polymer solutions can be based on a variety of different polymers and the concentration of them can be varied to create dilute or semi-dilute solutions. In dilute solutions the polymer molecules are diluted enough to not interact with each other, while in semi-dilute solutions, with a polymer concentration above their specific entanglement threshold c^* (Todorov and Morris 2002), the molecules interact or become entangled (Heller 2001). Higher concentration of low molecular weight polymers has proven to give a higher resolution of the separation of shorter RNA (approximately under the length of 1000 nt). However, longer RNA molecules require a lower concentration and longer polymers to be efficiently separated (Li, Liu et al. 2016). The viscosity of the separation medium is also of importance as this may affect the ease and reproducibility of the method. If the viscosity of the medium is too high it will require the application of high pressure and time to replace the medium (Boulos, Cabrices et al. 2008). Since a higher entanglement of polymers will also increase the viscosity of the solution, which will have a negative impact on the separation, a trade-off has to be made between high entanglement and low viscosity for optimal efficiency (Heller 1997).

2.3.1 Polymers

Various polymers have been explored as sieving media in the pursuit to find the optimal CGE separation qualities, which are commonly described as low viscosity, good sieving capacity and dynamic coating ability (Ke, Mo et al. 2010).

Linear polyacrylamide (LPA) has been widely used for the separation of DNA in CGE. Although it has been shown to have high sieving ability, the high viscosity of a solution containing the polymer is a big disadvantage in the high throughput, reproducible separation technique that CGE is promised to be (Boulos, Cabrices et al. 2008). Additionally, LPA lacks the ability of dynamically coating the inner wall of the capillary, resulting in a compulsory addition of an extensive pre-coating step to supress the EOF (Zhou, Yu et al. 2005).

Hydroxyethylcellulose (HEC) is a derivate of cellulose and a commonly mentioned polymer in literature relating to CGE. It is acknowledged to provide good sieving and is commercially available with several different molecular weights (Boulos, Cabrices et al. 2008). Several studies have been done to investigate the influence in sieving effects from different molecular weights and different concentrations. All conclude that the resolution of small RNA fragments/chains is best with the lower molecular weight HEC at a concentration over the entanglement threshold whereas an acceptable baseline separation of larger RNA molecules demands higher molecular weight of the polymer at a comparatively lower concentration (Heller 1997, Todorov, de Carmejane et al. 2001, Li, Liu et al. 2016). However, no matter how functional the sieving properties of HEC have proven to be, the fact that the polymer does not efficiently suppress EOF makes it insufficient as the optimal CGE polymer (Durney, Crihfield et al. 2015).

Polyvinylpyrrolidone (PVP) is another polymer that has gained attention in scientific CGE literature over recent years. PVP has the great advantage of low viscousity even at high molecular weights and is highly soluble in water, making sieving medium preparations more efficient and repeatedly homogeneous (Gao and Yeung 1998). The low viscosity of PVP can be illustrated by comparison with other CGE relevant polymers. A solution of 2% LPA (9 000 000 g/mol) at 25°C has a viscosity of 28 000 cP, 4% HEC (250 000 g/mol) at 25°C 3210 cP and finally 4% PVP (1 000 000 g/mol) at 25°C 15 cP (Boulos, Cabrices et al. 2008). Another highly beneficial quality of PVP is its ability to effectively suppress EOF by dynamic coating of the inner wall of capillaries. It has been seen that a high molecular weight of PVP has a positive effect on EOF suppression and that EOF decreases significantly with a higher concentration of PVP. Above 1% PVP concentration the EOF is almost completely suppressed. The efficient adhesion to the capillary wall is thought to be a result of hydrogen bonding to the protonated silanol groups of the capillary wall (Kaneta, Ueda et al. 2006). However, there is some controversy regarding PVP's ability as sieving medium. While some earlier articles report of a "relatively poor separation performance" (Chu and Liang 2002) more recently published work confirms the potential of PVP and its achievements in separating nucleic acid samples with good resolution (Mohamadi, Kaji et al. 2008, De Scheerder, Sparén et al. submitted).

Other polymers that frequently appear in literature are polydimethylacrylamide (PDMA), polyethylene oxide (PEO) and hydroxypropylmethylcellulose (HPMC). Of these, PDMA and PEO have dynamic coating ability and PDMA is recognized to have the best self-coating ability compared to the other sieving media with the same property. However, compared to LPA, HPMC gives lower separation resolution (Zhou, Yu et al. 2005). The viscosity of 2.5% PDMA (5 200 000 g/mol) is 33 300 cP at 25°C (Boulos, Cabrices et al. 2008). PEO has also been in the spotlight because of its favourable qualities, such as surface coating capability and relatively low viscosity, 1.5% PEO (600 kDa) has a viscosity of 1200 cP (Chung, Kim et al. 2014). Studies on PEO have indicated that RNA moves much faster in a solution of PEO compared to HEC solutions, which could be favourable for development of a time efficient method (Yamaguchi, Li et al. 2015). The polymer is commercially available in a wide range of molecular weights (Heller 1997). HPMC is like HEC a derivate of cellulose. It is not highly viscous and has at times been used to reduce adsorption of analyte to the capillary wall as well as to some extent to supress EOF (though not mentioned to have the dynamic coating ability as PVP, PEO and PDMA) (Chung, Kim et al. 2014). The addition of chemicals such as glycerol to a HPMC solution has had a positive effect on separation resolution (Heller 1997).

Despite the extensive research to find an optimal polymer to aid the separation of RNA and other biomolecules in CGE, as of this far not one has been found to fit all the criteria. Several studies have therefore aimed to find suitable copolymers or polymer mixtures, each with beneficial properties to enhance the separation process (Chu and Liang 2002). One polymer mixture that has been explored is the combination of HEC (250 000 g/mol) for its recognised sieving ability and the low viscous, self-coating capable polymer PVP (1 000 000 g/mol). The authors report a successful mixture of the two polymers as both improvement of resolution and suppression of EOF were achieved. Their final sieving medium composition had a total polymer concentration of 3.5% with 20.4% PVP compared to HEC (Boulos, Cabrices et al. 2008). Of the different copolymers that have been examined the combination of LPA and PDMA might be the intuitive first approach of study with their respective first-rate properties of sieving capacity and self-coating. Successful double-stranded (ds) DNA separation has been carried out using such sieving medium (Chu and Liang 2002).

Thermoresponsive sieving media is another possible approach to overcome some of the contradictive requirements for an optimal CGE method. The benefit of these polymers is their tuneable viscosity which can be changed with an increase or decrease in temperature. Replacement of the sieving medium can thereby be performed more efficiently at a certain temperature and then changing the temperature once the polymer matrix has been loaded into the capillary to utilize an increase in viscosity for increased separation ability. Examples of polymers that have been explored with this property are hydroxypropyl cellulose (HPC) and poly(*N*-isopropylacrylamide) (PNIPAM) (Zhou, Yu et al. 2005, Chung, Kim et al. 2014).

2.3.2 Additives

In addition to changing the polymer composition of the sieving medium, additional chemicals can be added to the solution to further enhance separation. Glycerol is frequently used to aid

the performance of polymer sieving media. It can form hydrogen bonds (Mohamadi, Kaji et al. 2008) with the effect of an increase in matrix density and thereby also an increase in viscosity (Kozlowski, Olejniczak et al. 2005). Mohamadi *et al.* (Mohamadi, Kaji et al. 2008) made a successful medium of 1.8% PVP with an addition of 10% glycerol where the separation efficiency was improved. Also, the addition of Mg^{2+} to PVP based sieving media has been shown to have a positive effect on separation. It has been suggested that the Mg^{2+} ions affect the PVP molecules in the medium by stabilizing ionic inter and intrachain bridges between the PVP molecules and thus improving the separation by creating a similar effect as an increase in polymer concentration (Mohamadi, Kaji et al. 2008).

Mg^{2+} is also frequently mentioned in the literature as a stabilizer of RNA secondary and tertiary structures. Even at addition of very low concentrations the binding of the positively charged metal ions to the RNA reduces the repulsion of the negatively charged phosphates and promote folding (Draper 2004). Addition of Mg^{2+} to the sieving medium therefore has a complex effect on both the properties of the medium as well as on the sample conformation.

Urea is another commonly used additive that has an effect on both the sieving medium and the sample to keep the RNA in a denatured single-stranded state. However, it has also a significant effect on the viscosity of the medium (Todorov and Morris 2002). The denaturing mechanism of urea on nucleic acids is described as the interaction with the exposed surface of bases, potentially by both hydrogen-bonding and stacking. It can also interact with the backbone and free ions to further hinder a secondary structure of the molecule (Lambert and Draper 2012). To achieve full denaturing effect urea is often added at concentration between 2 and 8 M (Skeidsvoll and Ueland 1996, Todorov, de Carmejane et al. 2001, Sumitomo, Sasaki et al. 2009). The high concentration of urea will outrival the hydrogen-bonding of the surrounding water molecules leaving the RNA molecules no longer in a water solution environment.

2.4 Sample preparation

Due to the presence of complementary fragments within an RNA strand, the same sequence can through intramolecular hydrogen bonds take on many different conformations. These structures are unpredictable and can cause peak shifts or band broadening (Gjerde, Hoang et al. 2009). Also multimers of RNA can be formed giving broader peaks. Denaturation of nucleic acid samples is described as essential for development of reproducible and reliable CGE methods with good separation resolution (Skeidsvoll and Ueland 1996, Todorov, de Carmejane et al. 2001, Sumitomo, Sasaki et al. 2009). In addition to the possible additives in the sieving medium for denaturing effects, the pre-treatment of RNA samples is highly important for improved separation. There are a few denaturing steps that are commonly applied to nucleic acid samples, including heating and addition of urea at high concentrations. By subjecting the RNA sample to high temperatures some of the hydrogen bonds creating these various conformations are broken and the structure variety is significantly reduced. However, some dimers/secondary structures of RNA will still be present also at high

temperatures. (Gjerde, Hoang et al. 2009). As previously mentioned, denaturing additives are also commonly applied to prevent the sample from taking on a number of different conformations. The most commonly used is urea which is often applied to both the sample and sieving medium for continuous denaturation of the sample throughout the separation (Todorov, de Carmejane et al. 2001, Lambert and Draper 2007, Li, Liu et al. 2016). However, there are also a number of other chemicals that have been evaluated for their effect on RNA and the resulting separation resolution. Formamide, formaldehyde, proline and acetic acid are a few of the alternative denaturants that have been explored. The chemical denaturants are often added to the sample prior to heating for optimal effect (Skeidsvoll and Ueland 1996, Lambert and Draper 2007, Sumitomo, Sasaki et al. 2009).

3 Material and methods

3.1 Equipment, samples and chemicals

All the materials used during the course of the project are described under the following section.

3.1.1 Capillary electrophoresis instrumention

The following subsections describe the instrumental setup for the experiments.

3.1.1.1 CE instrument

The CGE experiments were performed on an Agilent CE7100 system (Agilent Technologies, Santa Clara CA, USA). The CE auto sampler tray was set to 16°C with a LAUDA ecoLine RE106 thermostat. Detection of the analytes was done with a 260 nm detector Filter Assembly, CE7100-62700. The parameters of the methods and the resulting data collection were controlled by OpenLAB CDS ChemStation Edition software (Agilent Technologies, Santa Clara CA, USA).

Separations were carried out in a range between -12 kV and -30 kV depending on the capillary length. Current was not adjusted. Overall, the capillary temperature was kept at 25°C except for the experiments where the capillary temperature effect was evaluated. Sample injection was done by pressure either at 30 mbar or 80 mbar in the range of 9 to 43 seconds depending on capillary parameters and viscosity of the sieving medium.

3.1.1.2 Capillaries

Most capillaries used were bare fused silica capillaries from Agilent Technologies (Santa Clara CA, USA) with extended light paths. However, also a coated capillary from Agilent Technologies was used for some experiments. This capillary was cut using a capillary column cutter from Hewlett-Packard to get an effective length of 40 cm. The seven types of capillaries used during the course of the project are listed in *table 1*.

Table 1. Descriptions of the capillaries used for the experiments throughout the project.

ID (µm)	***Effective length (cm)***	***Total length (cm)***	***Extended light path (times wider than ID)***	***Part number***	***Type***
50	40	48.5	x3	G1600-60232	Bare fused
50	72	80.5	x3	G1600-62232	Bare fused
50	104	112.5	x3	G1600-64232	Bare fused
75	40	48.5	x2.7	G1600-60332	Bare fused
75	72	80.5	x2.7	G1600-62332	Bare fused
75	104	112.5	x2.7	G1600-64332	Bare fused
75	40	48.5	Standard	199-2602	Coated µSiL-DNA

3.1.1.3 Vials

Different types of vials were used for different solutions in the CGE analyses. Components such as MeOH, RNase free water, NaOH, coating solution and waste were kept in 2 ml glass crimp/snap top vial, part number: 5182-9697. Separation medium and refill were kept in 1 ml polypropylene (PP) crimp/snap top vials, part number: 5182-0567 and finally the samples in 250 µl PP cimp/snap top vials, part number 5188-2788. All bought from Agilent Technologies (Santa Clara CA, USA).

3.1.1.4 Commercial separation medium

The commercial separation medium used for the equipment experimental tests was DNF-265 RNA separation medium, normally used for the Fragment Analyzer Capillary Electrophoresis instrument (FACE), from Advanced Analytical Technologies (Ankeny IA, USA) with lot# 02BAYS03. Expiry date March 10th 2018. In this report called FACE sieving medium.

3.1.1.5 Separation gel 1

The sieving medium has optimized properties for separation of mRNA developed by De Scheerder *et al.* (De Scheerder, Sparén et al. submitted). The sieving properties are attributed to the presence of 1.32% PVP (M_w ~1 300 000) and enhanced by the addition of 10% glycerol. It also contains 15% HEPES buffer (adjusted to pH 7.5 with 1 M NaOH) and RNase free water. Due to its well documented composition and separation capability this sieving

medium composition was used as the basis for many of the sieving medium composition optimization trials.

3.1.2 RNA samples

The RNA ladder Ambion® RNA Century™-Plus Marker (Invitrogen) was bought from Thermo Fisher Scientific (Waltham MA, USA). It contains RNA transcripts of 100, 200, 300, 400, 500, 750 and 1000 bases in length at a concentration of 1 mg/ml, initially stored in -80°C and then -20°C short-term during usage. Several different lots of the ladder were used during the project: lot# 00497360 and lot# 00465792 pooled for the optimal capillary dimensions experiment and lot# 00494100 and 00518638 used for the remaining experiments with the ladder.

EPO mRNA, ~900 nt, was from TriLink BioTechnologies (San Diego CA, USA). Modifications from natural EPO mRNA consists of anti-reverse(ARCA) capped and fully substituted with 5-Methyl-C and Pseudo-U. Delivered as 1.00 mg/ml in RNase free H_2O. Lot #: T50-E01A. Initially stored in -80°C and then -20°C short-term. Aliquoted in smaller volumes to prevent excessive amounts of repeated freeze/thaw cycles.

3.1.3 Polymers

Polyvinylpyrrolidone (PVP) with M_w ~1 300 000 (lot# MKBN4168) and M_w ~360 000 (lot# SLBM9366V) bought from Sigma-Aldrich (St. Louis MO, USA).

2-Hydroxyethyl cellulose (HEC, M_w ~90 000, lot# MKBX0587V/ M_w ~250 000, lot# STBF4985V/ M_w ~720 000, lot# MKBT1583V) from Sigma-Aldrich (St. Louis MO, USA).

3.1.4 RNase free water

For all experimental work RNase free water was used to prevent the degradation of RNA by RNase. The RNase free water was produced through filtering Milli-Q water with a Biopak® Polisher from Merck KGaA (Darnstadt, Germany). Catalogue No: CD4FBI001, Lot #: F7CA89470, production date 28-Mar-2017 and installation date 15-May-2017.

3.1.5 Chemicals

Acetonitrile (C_2H_3N, >99.9%, lot# STBG0839V), formaldehyde solution (CH_2O, 36.5-38% in H_2O, lot# SZBG1180V), glycerol ($C_3H_8O_3$, ≥99%, lot# SHBH0231V), HEPES solution (N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid), 1 M pH 7.0-7.6, sterile-filters, BioReagent, suitable for cell culture, lot# SLBP0549V), sodium hydroxide (NaOH, ≥98%, ACS reagent, lot# SZBF1060V), sucrose ($C_{12}H_{22}O_{11}$, lot# BCBR8505L) and urea ($CO(NH_2)_2$, 99.0-100.5%, lot# SZBF2010V) were all bought from Sigma-Aldrich (St. Louis MO, USA).

Acetic acid (CH_3CO_2H, 99.7%, lot# N29C008) and magnesium chloride ($MgCl_2$, 1 M, lot# 1504001, Ambion) was from Thermo Fisher Scientific (Waltham MA, USA).

Methanol (CH_3OH, ≥99.9%, lot# STBG5347) was from Honeywell (Morris Planes NJ, USA).

3.2 Capillary Electrophoresis parameters optimisation

As an initial step towards improving the separation of shortmers in the mRNA sample the instrumental set up was investigated. The following section describes the experimental set ups.

3.2.1 Capillary dimensions

A total of six different capillaries were tested to evaluate the separation resolution achievable when varying the diameter and length of the capillary. The test included all bare fused silica capillaries given in *table 1*.

To elucidate the effect of the capillary characteristics on the separation, the instrumental procedures were adjusted for each capillary test. This to keep the injection volume and volt/cm same for all capillaries. The capillary described by De Scheerder (De Scheerder, Sparén et al. submitted) with 50 µm inner diameter (ID), 72 cm effective length was used as a reference for the capillary dimension test.

To get the injection volumes for all capillaries similar to the reference capillary *equation 1* was used. The same was done for the gel refill procedure. Also, the applied voltage was adjusted to allow comparison between the capillaries of different length. As the upper limit of applied voltage was theoretically breached by the longest capillaries (104 cm) these instead became the reference. They were assigned the highest possible applied voltage by the instrument (-30 kV) and the other capillaries run voltages was set from this. The viscosity value was set to 1 for all calculations, a representative number for water in room temperature.

Each capillary was tested according to the calculated specifications with FACE sieving medium and 0.1-1 kb RNA ladder as sample. The migration times of the resulting electropherograms were noted and the resolution between the peaks was calculated using *equations 2* and *3*.

3.2.2 Separation temperature

To investigate the effect of capillary temperature on the separation four different temperatures were evaluated. The temperatures 15°C (instrument minimum), 25°C, 40°C and 60°C (instrument maximum) were evaluated. The comparative study was carried out with a 50 µm, 40 cm capillary, chosen for time efficiency. A 0.1-1 kb RNA ladder was used as test sample and FACE sieving medium for the analyses. *Equation 2* and *3* were used to calculate the resolution of the separation as in the previous set of tests.

3.2.3 Precoating

A set of nine repeated runs were set up with an instrument method containing a precoating step with 1% PVP solution before the sieving medium refill step. A second set of nine runs were carried out with the same method but excluding the precoating step. The experiment was done with separation gel 1 as sieving medium and a sample of 0.5 mg/ml RNA ladder heated

at 80°C for 15 min. The standard deviation were calculated to give an indication of the reproducibility for each method.

3.2.4 Heavy wash

A capillary wash procedure was established included 2 min high pressure flush (5 bar) with RNase free water, 5 min flush (1 bar) with 0.1 M NaOH, 2 min flush rinse with RNase free water, 5 min flush with MeOH and a final 2 min flush rinse with RNase free water. Similarly to the precoating test the method excluding a precoating step was repeated for nine runs followed by heavy wash method also repeated for nine runs.

3.3 mRNA sample conformation

The following pretreatment procedures were explored for a beneficial conformation of the mRNA sample for CGE separation analysis.

3.3.1 Heating of sample

A batch of 0.5 mg/ml EPO mRNA was prepared by diluting a 1.00 mg/ml sample with RNase free water. 15 µl was then aliquoted into 6 Eppendorf tubes and put in the freezer. Each tube was individually taken out and thawed just before the start of its specific test to create a homogenous experiment setup without significant differences in delay time before analysis. The samples were thawed and mixed by pipetting before heating at 80°C in a thermoblock for 2, 5, 15, 30, 45 or 60 min. After heating, the tube was put directly on ice for 15 min and then quickly spun down in a centrifuge to be able to collect as much as possible when transferring the sample to a CE vial. The sample was mixed again before analysis.

3.3.2 Chemical additives in sample

3.3.2.1 Urea

30 µl of 0.5 mg/ml RNA ladder sample and 30 µl 0.5 mg/ml EPO mRNA sample were prepared by diluting a portion of the original 1 mg/ml batch samples with an equal part of RNase free water in new Eppendorf tubes. Approximately 7 mg of urea powder, making the final urea concentration around 3.2 M in each sample, was added to each sample. The sample tubes were then heated at 80°C for 15 min and then put directly on ice before being quickly spun down and transferred to new CE sample vials. The samples were then analysed using separation gel 1.

3.3.2.2 $MgCl_2$

1 µl 1 M $MgCl_2$ was added to 50 µl of 0.5 mg/ml RNA ladder and EPO mRNA respectively and mixed by pipetting, resulting in a 20 mM $MgCl_2$ concentration in both tubes. The sample tubes were then treated with the usual procedure; 80°C for 15 min, ice 15 min, quickly spun down in centrifuge, transferred to CE sample vials and analysed with separation gel 1.

3.4 Sieving medium optimisation

The basis for all sieving medium investigations during the project were based on the composition of separation gel 1 previously developed at the department by De Scheerder *et al.* (De Scheerder, Sparén et al. submitted).

3.4.1 Polymers

Following section describes the explored sieving media compositions to enhance separation.

3.4.1.1 PVP

PVP with a molecular weight of ~1 300 000 was used for the majority of the optimisation experiments as this was the sieving basis for the previously developed separation gel 1. The polymer concentration was set to 1.32% to enable justified comparisons between different sieving medium variants. Experiments with increased concentration of PVP (2% and 5%) in an otherwise unchanged composition of separation gel 1 were also performed. Additionally, a PVP of smaller molecular weight, ~360 000, was also evaluated.

3.4.1.2 HEC

Also HEC, in a variety of molecular weights, was explored as a potential polymer for enhanced separation. Experiments were performed using HEC with a molecular weight of 90 000 Da, 250 000 Da and 720 000 Da respectively using the otherwise same composition as separation gel 1. The alternative sieving media then contained 1.32% of either HEC 90 kDa, 250 kDa or 720 kDa, 10% glycerol, 15% HEPES (pH 7.5) and RNase free water. Another sieving solution variant with 720 kDa HEC excluding glycerol, to allow for lower viscosity, was also investigated. CGE analyses for all three molecular weights of HEC were carried out with a bare fused silica capillary (ID 50 µm, effective length 40 cm) both with and without a 1% PVP precoating step included in the method. In addition, a coated µSiL-DNA capillary (ID 75 µm, effective length 40 cm) was used to exclude any effect of PVP on the separation while maintaining a sufficiently supressed EOF. The injection parameters were increased for the coated capillary (compared to the calculated comparable parameters used in for the uncoated capillary tests) to 80 mbar, 30 s to gain higher intensity of the peaks.

3.4.1.3 PVP/HEC

Sieving media containing a mixture of polymers were also investigated. Combinations of PVP and HEC were evaluated at different relative and total concentrations. The initial approach was inspired from (Boulos, Cabrices et al. 2008) and resulted in a mix of PVP (M_w ~1.3 MDa) and HEC (250 kDa) at a concentration of 0.714% and 2.786% respectively and a total polymer concentration of 3.5% with otherwise the same components as in separation gel 1. Another similar solution was prepared with exclusion of glycerol for a lowered viscosity. Additionally, other proportions and total polymer concentrations were evaluated, including 75/25, 50/50 and 25/75 proportions of PVP (1.3 MDa)/HEC (250 kDa) with a total polymer concentration of 1.32% and 1.32% PVP with addition of 0.5%, 1.0% and 1.5% HEC (250 kDa). All with the basis of separation gel 1 including 10% glycerol, 15% HEPES (pH 7.5)

and RNase free water. The sieving media were evaluated using an RNA ladder and a method excluding any precoating step.

3.4.2 Chemical additives in sieving medium

The separation gel 1 developed at AstraZeneca (De Scheerder, Sparén et al. submitted) was used for the additive experiments to have complete control of the composition and be able to control the percentage and concentration of all additives in the sieving medium. A stock solution excluding part of the RNase free water volume was used for all additive experiments.

3.4.2.1 Magnesium

The $MgCl_2$ sieving media solutions were prepared with 6.636 ml of the sieving medium additive stock and adding the appropriate amount of $MgCl_2$ (1 M) to the concentration of interest for each experiment (e.g. 20 µl for 2 mM and 200 µl for 20 mM). RNase free water was then added to reach 10 ml (3.344 ml for 2 mM $MgCl_2$ and 3.164 ml for 20 mM $MgCl_2$). The sieving media containing the additive were homogenised by carefully turning the corresponding Falcon tube over ≥20 times.

3.4.2.2 Acetonitrile

10 ml sieving medium consisting of 20% acetonitrile was prepared by taking 6.636 ml of the sieving medium additive stock solution, adding 2 ml Acetonitrile (>99.9%) and an additional 1.364 ml RNase free water. The solution was mixed by carefully turning over the Falcon tube ≥20 times.

3.4.2.3 Sucrose

1 g of sucrose was added to 6.636 ml sieving medium additive stock and 2.364 ml RNase free water, resulting in a 10% sucrose additive sieving medium. The solution was mixed by using a magnetic stirrer until homogeneous.

Another sucrose sieving medium was prepared by replacing the glycerol in the separation gel 1 with sucrose. The final composition of the sieving medium was then 1.32% PVP, 10% sucrose, 15% HEPES (pH 7.5) and RNase free water. A magnetic stirrer was used to homogenize the solution.

3.4.2.4 Formaldehyde

For a 20% (of total volume) formaldehyde sieving medium 2 ml of formaldehyde was added to 6.636 ml sieving medium additive stock and 1.264 ml RNase free water. The solution was then mixed by using a magnetic stirrer.

3.4.2.5 Acetic acid

A sieving medium containing 2 M acetic acid was tested for potentially increased separation ability. The solution was prepared by adding 1.149 ml acetic acid to 6.636 ml sieving medium additive stock and 2.215 ml RNase free water. A magnetic stirrer was used to mix the solution.

3.4.2.6 Urea

2.4024 g of urea was added to a 10 ml aliquot of separation gel 1 giving a final concentration of approximately 3.3 M to the sieving medium. The solution was mixed using a magnetic stirrer for approximately 1.5 hours.

3.5 Optimised CGE separation parameters test

Based on the results of each optimisation test the most promising parameters and sieving medium compositions were all gathered into one CGE analysis set up. The final experimental set up consisted of a capillary of ID 75 µm and effective length 104 cm, cassette temperature set at 25°C, sieving medium composed of 1.32% PVP (1.3 MDa) + 1.0% HEC (250 kDa), 10% sucrose, 15% HEPES of pH 7.5 and RNase free water. The RNA samples were prepared by heating at 80°C for 15 min followed by cooling on ice for 15 min. The analysis was performed with a method containing a heavy wash procedure (2 min high pressure flush (5 bar) with RNase free water, 5 min flush (1 bar) with 0.1 M NaOH, 2 min flush rinse with RNase free water, 5 min flush with MeOH and a final 2 min flush rinse with RNase free water) prior to capillary gel refill and excluding a precoating step. The voltage was set to -30 kV and injection was performed using 30 mbar for 22 s.

4 Results and discussion

4.1 Optimising Capillary Electrophoresis parameters

Following section describes the findings of the optimisation experiments concerning the capillary electrophoresis parameters.

4.1.1 Capillary dimensions

After a comparative study of the capillaries the resolution data in *table 2* clearly indicate that the capillary with ID 75 µm and effective length of 104 cm contribute to a more advantageous CGE separation for the purpose of enhanced pre-main peak separation (<800 nt). Overall, the longer capillaries give higher resolution for the same separation. The additional length gives the sample more time in the capillary and thereby also more time for each fragment to separate from each other. Interestingly, a wider ID seems to enhance the resolution compared to the capillary of same length with a narrower ID. This was a somewhat surprising result as better resolution is expected with a thinner capillary where the sample would likely be less broadened due to thermic gradients and therefore result in more distinguishable peaks.

Table 2. Resolution data between each pair of subsequent peaks for the tested capillaries. R = resolution.

ID (µm)	Effective length (cm)	R 1-2	R 2-3	R 3-4	R 4-5	R 5-6	R 6-7
50	40	5,36	2,62	2,02	1,63	4,45	0,230
50	72	7,51	3,77	2,85	2,49	6,56	0,395
50	104	8,96	5,12	4,09	3,29	9,44	0,560
75	40	6,40	3,05	2,43	1,76	4,59	0,228
75	72	9,49	4,72	3,89	3,03	8,55	0,391
75	104	11,2	6,67	5,56	3,74	11,6	0,559

Though the separation was improved by the dimensions of the 75 µm, 104 cm capillary (*figure 2*) the increase in analysis time might not be universally optimal, for instance if a large number of samples must be analysed in a limited time. However, in the case of a thorough and specific analysis it could provide the necessary resolution for closely migrating fragments.

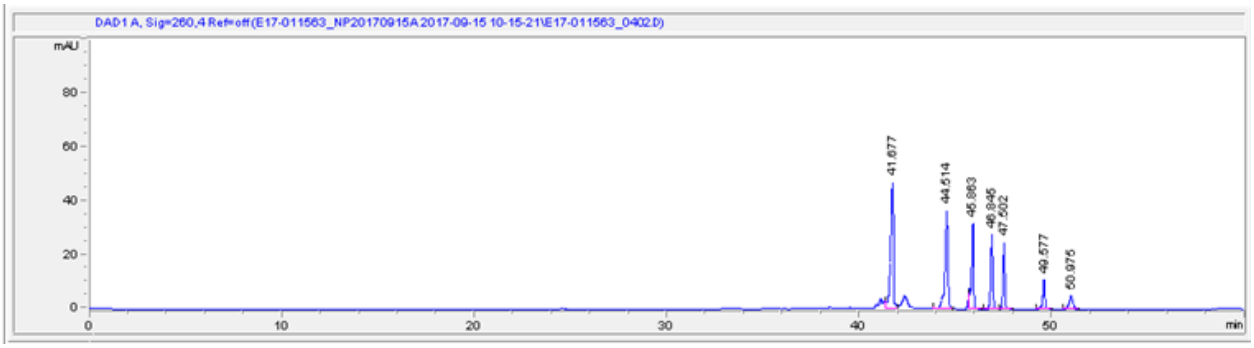

Figure 2. RNA ladder 1 mg/ml, heated 80°C for 2 min, in FACE sieving medium in **ID 75 µm, effective length 104 cm capillary** at 25°C. Injection parameters 30 mbar, 23 s. Applied voltage: -30 kV.

Electropherograms of all tested capillaries can be seen in *appendix A.*

4.1.2 Separation temperature

The different capillary cassette temperatures gave significant differences in migration time for the sample as can be seen in *figure 3*. The peaks of the 60°C analysis migrate through the

capillary much faster than the analyses at lower temperatures. Furthermore, the peaks have a higher intensity. However, due to the decrease in retention time for the higher temperature the separation between the peaks is not as good compared to for the lower temperatures. Though none of the analyses show baseline separation between the peaks, at the lower temperatures the time difference between the peaks increased. When comparing the 60°C analysis with the one at 40°C it can be observed that another peak is emerging right after the first peak. This peak is then further separated at the lower cassette temperatures. The peaks of the separation at 15°C are comparatively low in intensity while still at a similar width. Nevertheless, this separation reveals more peaks than the other. Though some peaks appear wider compared to the corresponding at higher temperatures this can in some cases be the result of the start of a further separation of analytes within the peak in question, such as in the case of the first peak. This is likely a result of the change of viscosity for the sieving medium at different temperatures, higher temperatures giving the medium a lower viscosity. Likewise giving the sieving medium a higher viscosity at lower temperatures, the migration time increase thus giving the molecules more time for a more extensive separation. The 25°C separation displayed a relatively high peak intensity, with enhanced separation from the higher temperatures while still achieving the separation in a reasonable amount of time. However, the different cassette temperatures can have advantages and disadvantages in different situations. For example, an increased cassette temperature can be advantageous in the case of using a longer capillary to reduce the time of the separation or for a polymer sieving medium with a higher viscosity to reduce the viscosity of the matrix for a more efficient refill of the capillary.

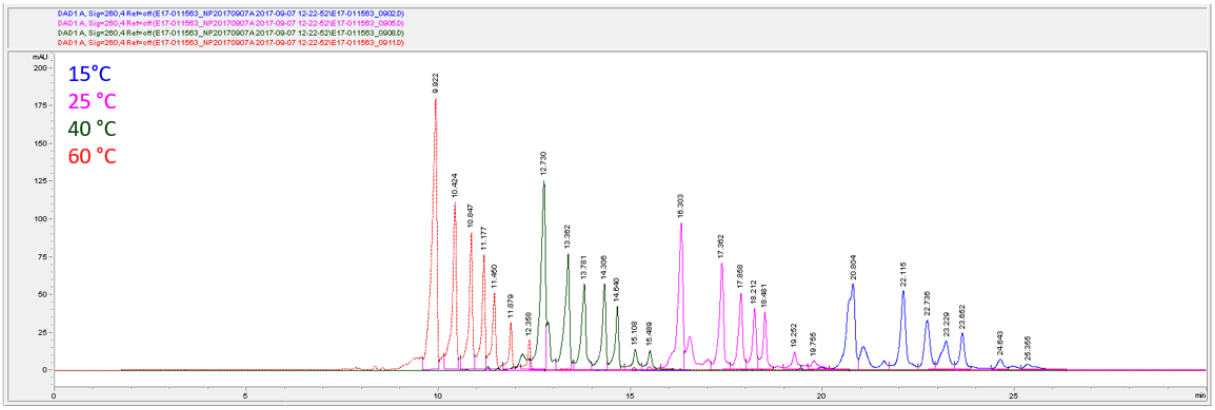

Figure 3. RNA ladder 1 mg/ml, heated 80°C for 2 min, in FACE sieving medium in ID 50 µm, effective length 40 cm **capillary at 15°C, 25°C, 40°C and 60°C respectively**. Injection parameters 80 mbar, 18 s. Applied voltage: -13 kV.

Further studies should include a set current for all analyses at different temperatures as this could influence the migration time to become more similar for the analyses. This would then provide an interesting investigation of the influence of the separation temperature at a similar migration time provided by the set current.

4.1.3 Precoating and prewash evaluation

The following tests were set up to study the effect of a precoating step, with the goal of achieving a more reproducible analysis method.

4.1.3.1 Precoating

The result presented in *table 3* indicate that when using separation gel 1, a precoating step with a simple 1% PVP water solution is not beneficial for the reproducibility of migration times. This could be because of PVP's good coating ability it is enough to simply fill the capillary with the sieving medium to effectively supress EOF and create a stable environment for separation. When adding a precoating step before the sieving medium refill of the capillary the use of an additional step in the preparative procedure of the capillary seems to be more disruptive of its inner environment than helpful. The standard deviation is lowered five-fold when excluding the precoating step.

Table 3. Effect of precoating and no precoating methods for repeatability of migration times (1st set). T = migration time and the number specifies the peak (1 = 100 nt, 2 = 200nt, 3 = 300 nt, 4 = 400 nt, 5 = 500 nt, 6 = 750 nt, 7 = 1000 nt).

1st set	***Precoat (migration times in min)***							***No precoat (migration times in min)***						
Calculations	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**
Average	16,4	17,5	18,0	18,4	18,6	19,4	20,0	16,2	17,3	17,8	18,2	18,5	19,3	19,8
Standard deviation	0,106	0,104	0,103	0,101	0,100	0,099	0,104	0,022	0,021	0,021	0,021	0,021	0,021	0,023
Relative standard deviation (%)	0,643	0,597	0,572	0,550	0,539	0,509	0,521	0,133	0,123	0,116	0,114	0,112	0,111	0,118

4.1.3.2 Heavy wash

Following the outcome of the precoating experiment (section *4.1.3.1*) it was hypothesised that a build-up of polymer layers might be the cause of problem for the higher deviation in migration times for the peaks. To completely wash out any residues from the previous run would then be beneficial. A follow up investigation was done, once again performed with the method excluding the precoating step but with the addition of a heavy wash of the capillary before each run. The procedure was set up to make sure that the capillary was completely free of any sieving medium residues from the run before. When using the heavy wash the

precision in migration time is improved by a factor ten compared to the same method excluding the wash (see *table 4*).

Table 4. Effect of no precoating and heavy wash, no precoating methods for repeatability of migration times (2nd set). T = migration time and the number specifies the peak (1 = 100 nt, 2 = 200nt, 3 = 300 nt, 4 = 400 nt, 5 = 500 nt, 6 = 750 nt, 7 = 1000 nt).

2nd set	*No precoat (migration times in min)*							*Heavy wash, no precoat (migration times in min)*						
Calculations	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**
Average	16,2	17,3	17,8	18,2	18,5	19,3	19,8	16,2	17,2	17,7	18,1	18,4	19,2	19,7
Standard deviation	0,081	0,092	0,098	0,102	0,106	0,117	0,124	0,007	0,008	0,009	0,009	0,010	0,011	0,011
Relative standard deviation (%)	0,496	0,532	0,552	0,561	0,573	0,605	0,628	0,042	0,047	0,049	0,051	0,052	0,057	0,058

Moreover, there is a significant difference in the deviation numbers from the no precoating method of the 1st (*table 3*) and 2nd (*table 4*) set of analyses. Looking closer on the electropherograms of each set reveals that one analysis is unusually deviating in the 2nd set. Excluding this from the standard deviation calculations lowers the standard deviation to around 0.04 for all peaks. This is however still above what is achieved for at 1st set. One hypothesis for this is that it has to do with the state of the sample. From this and earlier experiments it has been seen that the intensity of the peaks in identically performed analyses decreases over time until finally settling around a lower intensity after several repeated runs. This could then have a certain effect on the migration times as the peak shapes would differentiate some over time, explaining the different result of the method without precoating in set 1 and 2. Another hypothesis for the decrease in intensity could be that the inner surface of the capillary had been saturated, possibly by RNA sample.

Electropherograms and further visualisation of the difference in migration times can be seen in *appendix B.*

4.2 Investigation of optimal conformation of mRNA for CGE separation

As single stranded RNA molecules tend to fold into a variety of secondary structures and multimers it is beneficial to find a pre-treatment procedure for a more reproducible and high

resolution analysis method. The following section describes the experimental attempts of pretreatment of the RNA samples to find an optimal sample conformation for CGE analysis.

4.2.1 Heating of sample

An experiment was set up to provide a better understanding of how a mRNA sample is affected by heating. The standardised heating of mRNA samples described in the literature (De Scheerder, Sparén et al. submitted) is 80°C for 2 min with the aim to fully denature the RNA. However, it is not completely clear for how long a sample should be exposed to the heat for the best denaturing result without causing too much degradation.

Figure 4 and *5* demonstrate that after 2 and 5 min of heating at 80°C there are secondary structures or multimers present in the sample as there is a slight peak tail after the main peak. In *figure 6* with 15 min of heating the post-main peaks have disappeared, which suggests that the alternative secondary structures and multimers seen in the previous figures have been denatured. However, a slight decrease in main peak intensity can also be observed and the pre-main peaks have intensified compared to in the two previous figures, indicating that the RNA sample has been somewhat degraded. After 30 min at 80°C, demonstrated in *figure 7*, the post-main peak tail is still absent. The intensity of the main peak has decreased further, and the pre-main peak are further increased. *Figure 8* and *9*, illustrating the sample after 45 and 60 min of heating respectively, show that the mRNA sample has been fully degraded. The result of this experiment suggests that heating the sample at 80°C for 15 min is beneficial for separation studies where secondary structures and multimers need to be diminished.

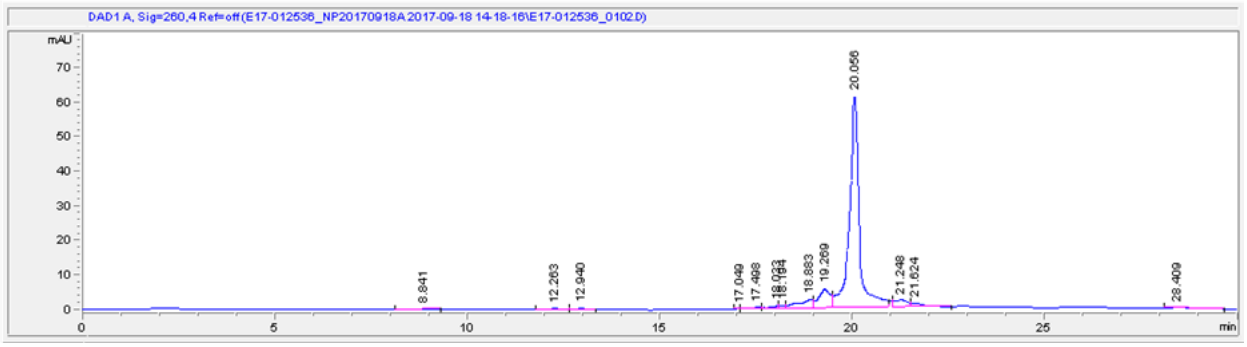

Figure 4. EPO mRNA 0.5 mg/ml, **heated 80°C for 2 min**, in FACE sieving medium in ID 50 µm, effective length 40 cm capillary at 25°C. Injection parameters 80 mbar, 18 s. Applied voltage: -13 kV.

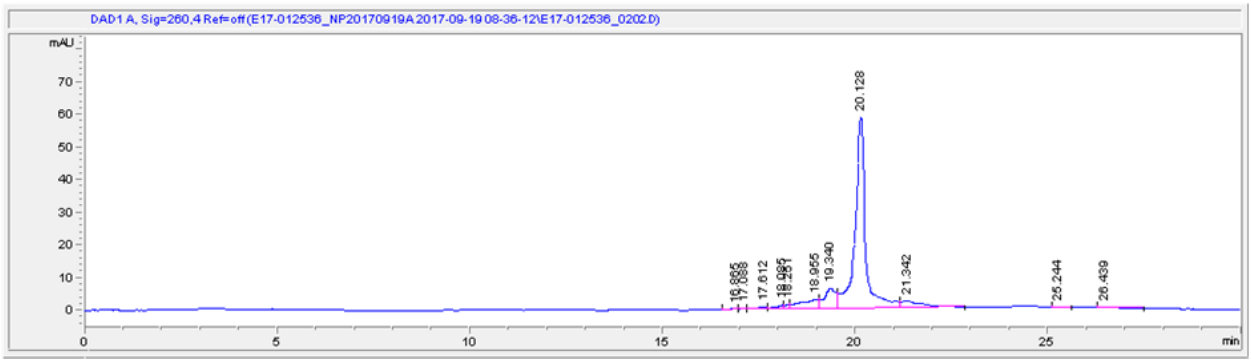

Figure 5. EPO mRNA 0.5 mg/ml, **heated 80°C for 5 min**, in FACE sieving medium in ID 50 µm, effective length 40 cm capillary at 25°C. Injection parameters 80 mbar, 18 s. Applied voltage: -13 kV.

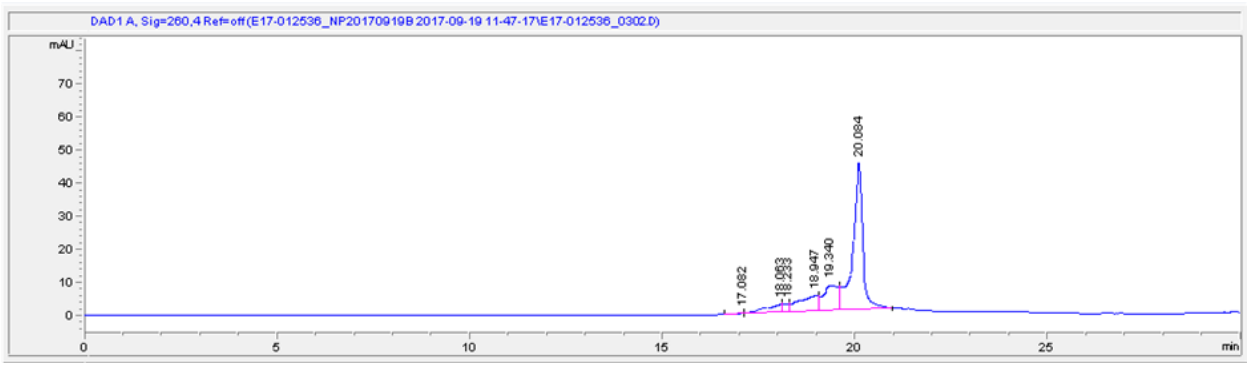

Figure 6. EPO mRNA 0.5 mg/ml, **heated 80°C for 15 min**, in FACE sieving medium in ID 50 µm, effective length 40 cm capillary at 25°C. Injection parameters 80 mbar, 18 s. Applied voltage: -13 kV.

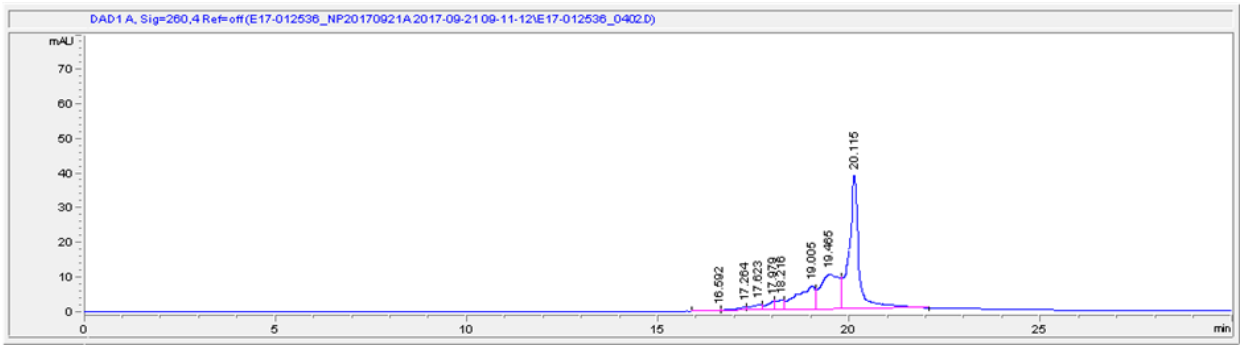

Figure 7. EPO mRNA 0.5 mg/ml, **heated 80°C for 30 min**, in FACE sieving medium in ID 50 µm, effective length 40 cm capillary at 25°C. Injection parameters 80 mbar, 18 s. Applied voltage: -13 kV.

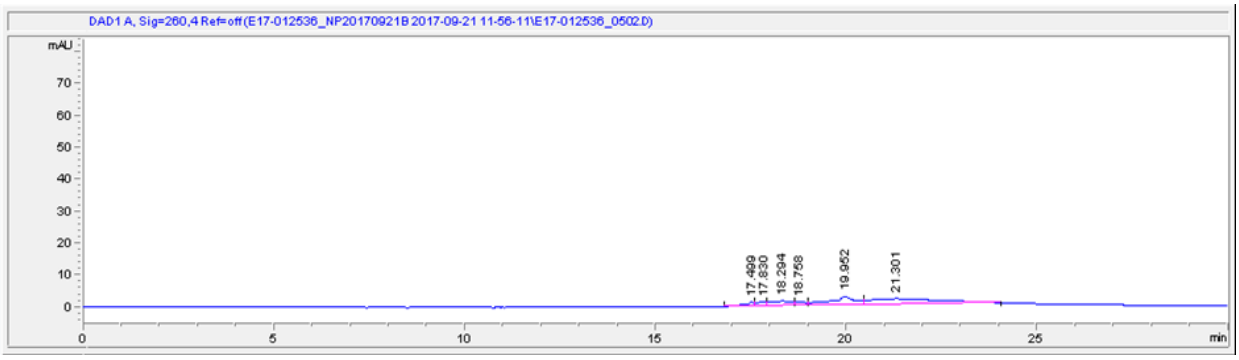

Figure 8. EPO mRNA 0.5 mg/ml, **heated 80°C for 45 min**, in FACE sieving medium in ID 50 µm, effective length 40 cm capillary at 25°C. Injection parameters 80 mbar, 18 s. Applied voltage: -13 kV.

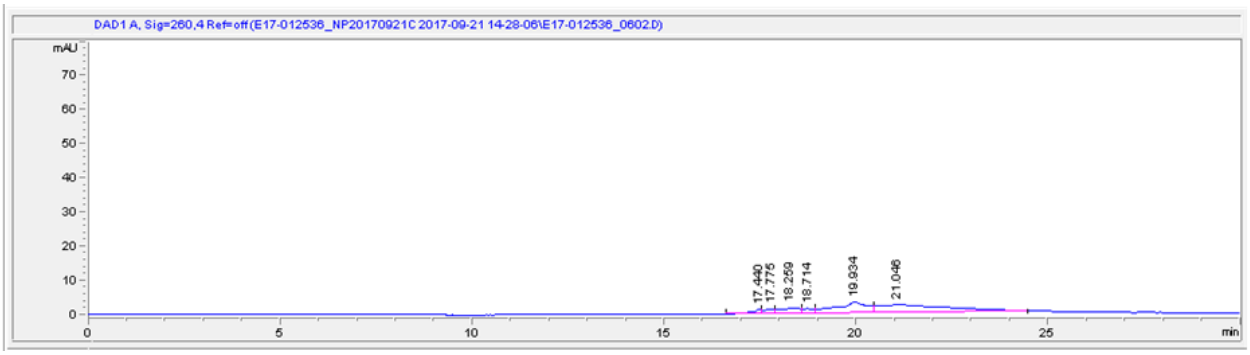

Figure 9. EPO mRNA 0.5 mg/ml, **heated 80°C for 60 min**, in FACE sieving medium in ID 50 µm, effective length 40 cm capillary at 25°C. Injection parameters 80 mbar, 18 s. Applied voltage: -13 kV.

4.2.2 Sample additives

With foundation in the literature (Todorov, de Carmejane et al. 2001, Draper 2004, Lambert and Draper 2007, Li, Liu et al. 2016) two chemicals were investigated as sample additives with ambition to find an interaction which would enhance the resolution.

4.2.2.1 Urea

Looking at the result of the addition of urea to the RNA ladder in *figure 10* it is visually difficult to distinguish it from an analysis of an ordinary RNA ladder sample without additives (see *figure 12*). However, the EPO mRNA analysis with approximately 3.2 M urea as additive seen in *figure 11* is distinguishable from an ordinary analysis, see *"4.4.1 Commercial sieving medium vs. separation gel 1"*, as the pre-main peaks appear spikier than usual. Which could have benefits for detection and distinguish fragments from of other closely migrating fragments.

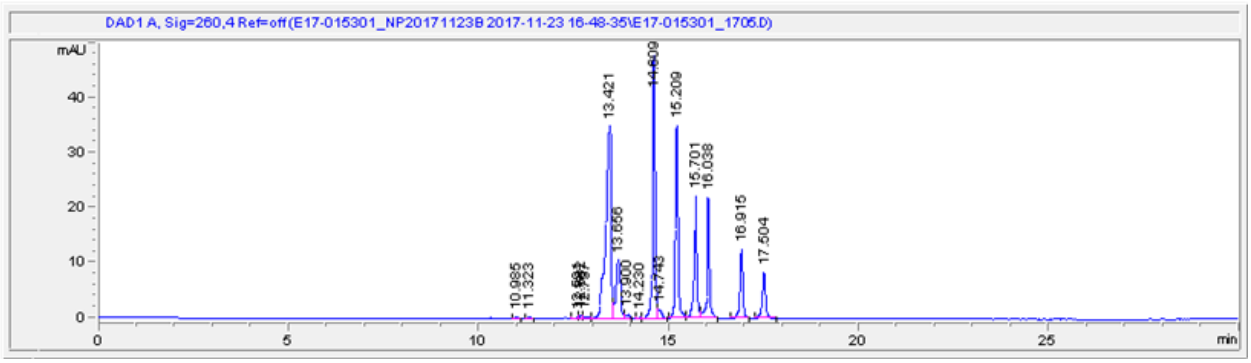

Figure 10. **RNA ladder 0.5 mg/ml + 3.2 M urea**, heated 80°C for 15 min, in separation gel 1 in ID 50 µm, effective length 40 cm capillary at 25°C. Injection parameters 80 mbar, 18 s. Applied voltage: -13 kV.

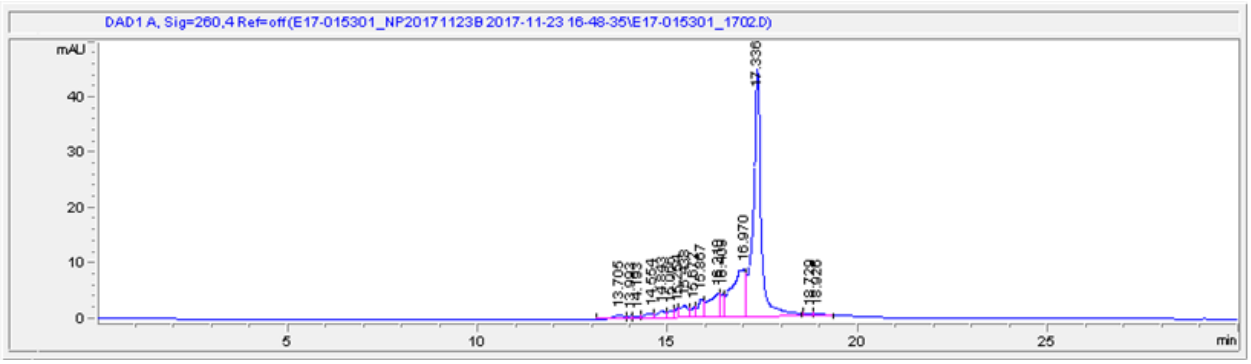

Figure 11. **EPO mRNA 0.5 mg/ml + 3.2 M urea**, heated 80°C for 15 min, in separation gel 1 in ID 50 µm, effective length 40 cm capillary at 25°C. Injection parameters 80 mbar, 18 s. Applied voltage: -13 kV.

A comparison of untreated EPO mRNA and EPO mRNA + 3.2 M urea can be seen in *appendix C*.

4.2.2.2 Magnesium

No peaks were detected from the analysis of the EPO mRNA sample with 20 mM $MgCl_2$ as additive (see *appendix D*). One possibility for this result is that the Mg^{2+} has bound to the RNA resulting in a neutrally or even positively charged speced that no longer migrate towards the detection end of the capillary.

No result was obtained for the RNA ladder with 20 mM $MgCl_2$ as the system was contaminated after the initial analysis of the EPO mRNA with 20 mM $MgCl_2$ as additive. The system was not fully functional until the capillary and all vials present had been replaced.

The only effect from $MgCl_2$ as a sample additive that could be observed was that of the control analyses performed after the actual test. As these are performed under the normal conditions of separation gel 1 and an untreated (only heat treated for denaturation) RNA ladder sample no deviation from the standard result is expected if the system is unharmed from the experimental analysis. Yet in this case the standard separation is poorer than normal as several peaks have lost the baseline separation otherwise present. Even when all vials were exchanged the poor separation persisted. This implies that the $MgCl_2$ had contaminated the

capillary wall and/or other vital parts of the instrument and could not be removed even by rinse washes of the capillary. From the severe implication of the relatively small $MgCl_2$ addition into the system it can be concluded that this additive does not provide the required separation effects but instead creates an unfavourable and irreversible separation environment.

4.3 Sieving medium composition

The result of the evaluated sieving medium composition is describes in the following section.

4.3.1 FACE sieving medium vs. separation gel 1

Separation analyses performed with separation gel 1 and FACE sieving media respectively for a visualisation of the difference in separation performance (*figures 12* and *13*). *Table 5* presents the resolution of the RNA ladder peaks using the two different sieving media.

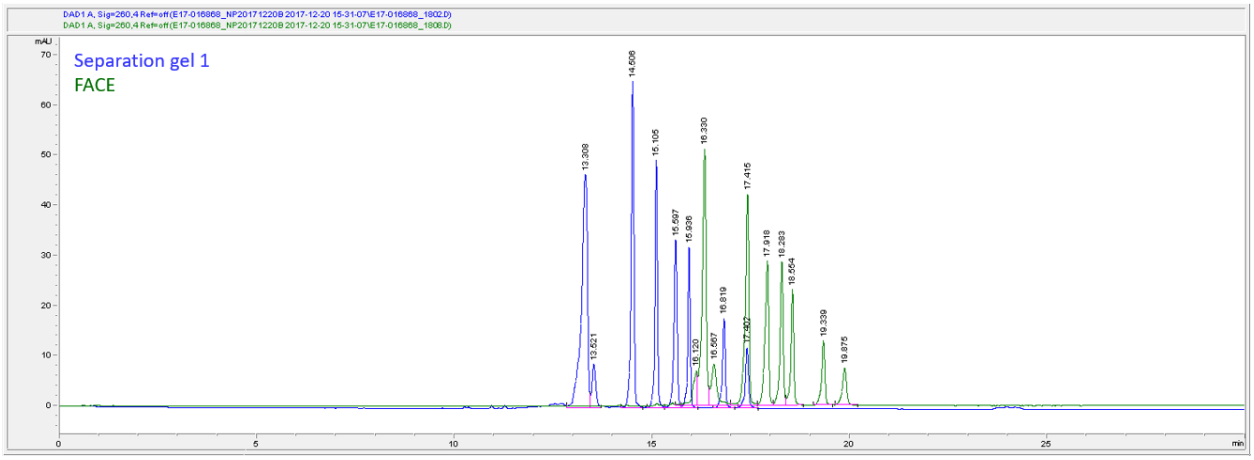

Figure 12. RNA ladder 0.5 mg/ml, heated 80°C for 15 min, in **separation gel 1 and FACE sieving medium** in capillary of ID 50 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar, 18 s. Applied voltage: -13 kV.

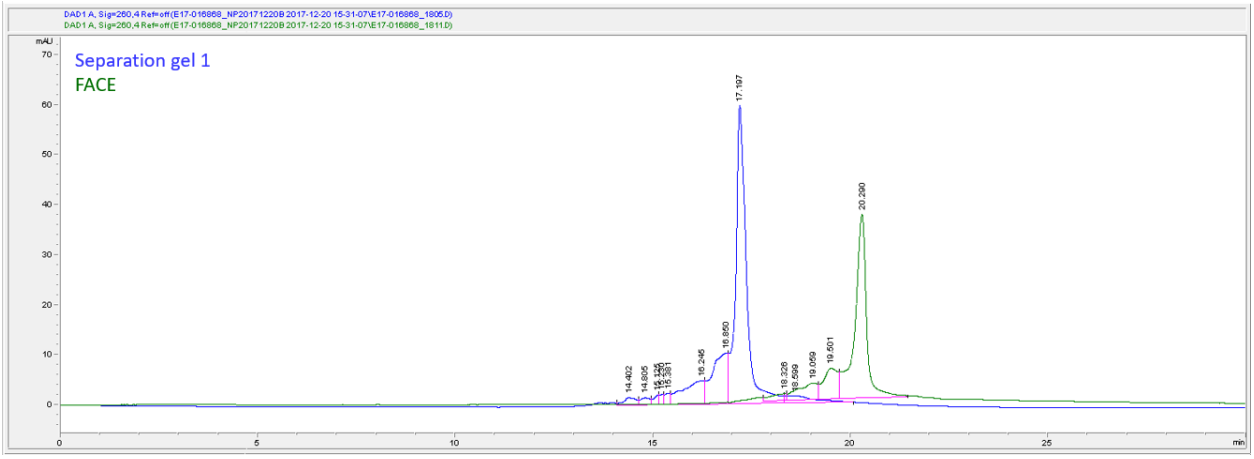

Figure 13. EPO mRNA 0.5 mg/ml, heated 80°C for 15 min, in **separation gel 1 and FACE sieving medium** in capillary of ID 50 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar, 18 s. Applied voltage: -13 kV.

Table 5. Resolution data for analyses with separation gel 1 and FACE sieving medium respectively. Capillary with ID 50 µm, effective length 72 cm at 25°C. T = migration time and the number specifies the peak (1 = 100 nt, 2 = 200nt, 3 = 300 nt, 4 = 400 nt, 5 = 500 nt, 6 = 750 nt, 7 = 1000 nt).

Sieving medium	R 1-2	R 2-3	R 3-4	R 4-5	R 5-6	R 6-7
Separation gel 1	5,964	4,935	3,740	2,622	7,205	0,233
FACE	6,343	3,077	2,402	1,954	5,427	0,214

4.3.2 Polymers

The investigation of suitable polymers was narrowed down to two potential polymers for further investigation. Following section describes the result of further examination with PVP and HEC polymers.

4.3.2.1 PVP

Comparing the analysis using a sieving medium of 5% PVP and one of 1.32% as shown in *figure 14*, reveals that a higher concentration of the polymer drastically increases the separation of the peaks. However, this is at the cost of a more than doubling of the separation time and a severe drop in intensity. With this higher entanglement of the polymer and increase in migration time bandbroadening might become an issue. Also, the decrease in intensity decreases the ability to detect fragments of lower concentration in the sample. The prolonged separation time suggests that the viscosity of the 5% PVP sieving medium is significantly higher than that of the 1.32%. This would then affect the sample volume injected which would explain the decrease of intensity.

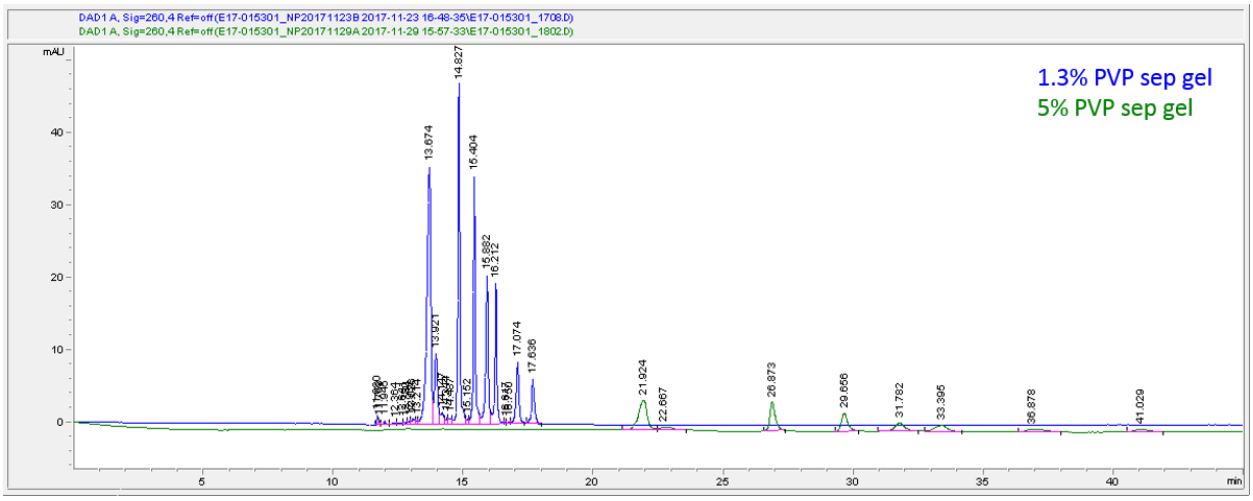

Figure 14. RNA ladder 0.5 mg/ml, heated 80°C 15 min, in sieving medium containing **1.32% PVP or 5% PVP**. Capillary of ID 50 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar 18 s. Applied voltage: -13 kV.

Using 2% PVP (*figure 15*) also prolongs the migration time and lowers the intensity of the peaks. However, this increase in polymer concentration only prolongs the migration time by approximately 2 min with only slightly decreased intensity once again caused by the increase in viscosity. It has however contributed to an improved separation where the 4th and 5th peak of the sample are now baseline separated compared to when using the ordinary concentration.

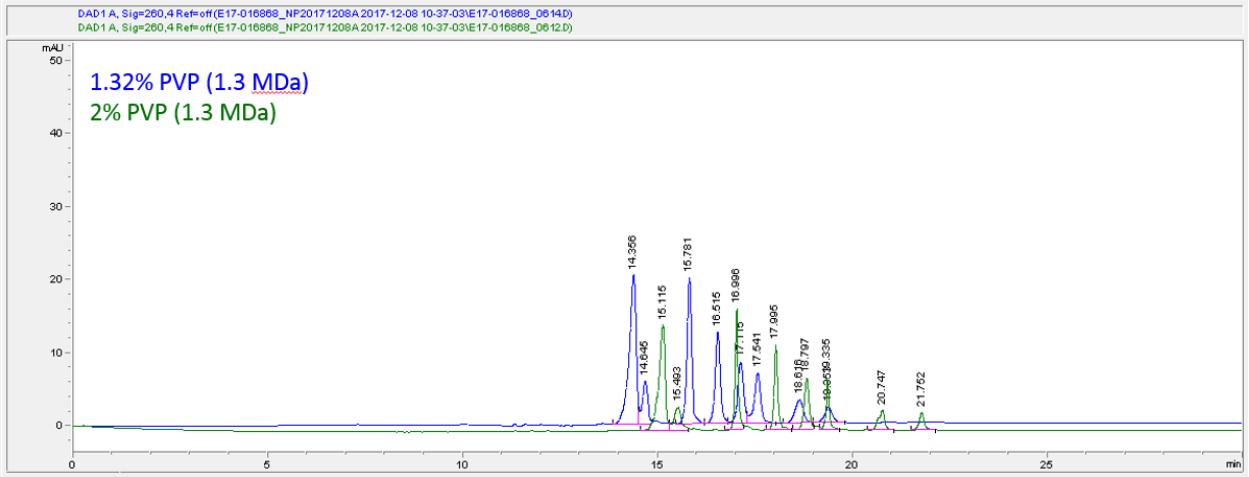

Figure 15. RNA ladder 0.5 mg/ml, heated 80°C 15 min, in sieving medium containing **1.32% PVP or 2% PVP.** Capillary of ID 50 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar 18 s. Applied voltage: -13 kV.

For the PVP of a significantly lower molecular weight (*figure 16*) the separation follows a very similar pattern as for the 1.3 MDa PVP. For the standard concentration of 1.32% the migration time is fairly quicker and the separation appears to be very similar to that of the larger molecular weight. Nevertheless, studying the 2% PVP solution the migration time appear to increase more drastically with increased concentration compared to for the larger molecular weight media. The 360 kDa PVP was determined to not have any significantly different or beneficial effect on the separation and was not further investigated.

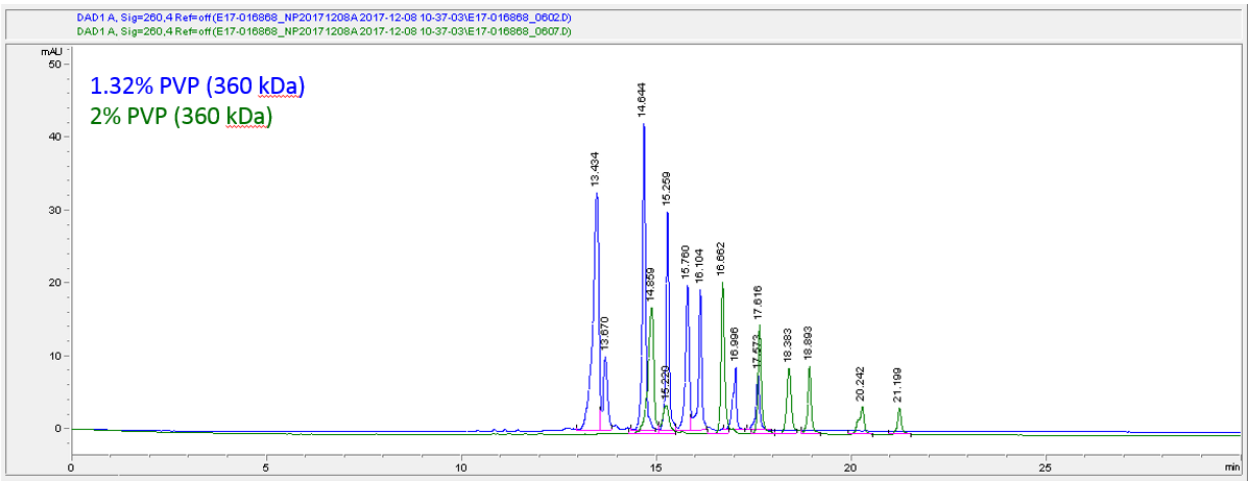

Figure 16. RNA ladder 0.5 mg/ml, heated 80°C 15 min, in sieving medium containing **1.32% PVP (360 kDa) compared to in 2% PVP (360 kDa).** Capillary of ID 50 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar 18 s. Applied voltage: -13 kV.

4.3.2.2 HEC

Figures 17 and *18* illustrate the separation achieved with HEC with a molecular weight of 90 kDa. Using an uncoated silica capillary with a 1% PVP precoating step give a sufficient separation of the RNA ladder. However, the peak shape shows a tendency of tailing, which could make baseline separation more difficult. The same tendency is observed for the separation in the permanently coated capillary and in this case the separation is worse than in the previous case, being even further from complete baseline separation. To achieve a detectable height of all peaks the injection parameters had to be increased from initial trials. This slight overload of sample likely then contributed to the poorer baseline separation.

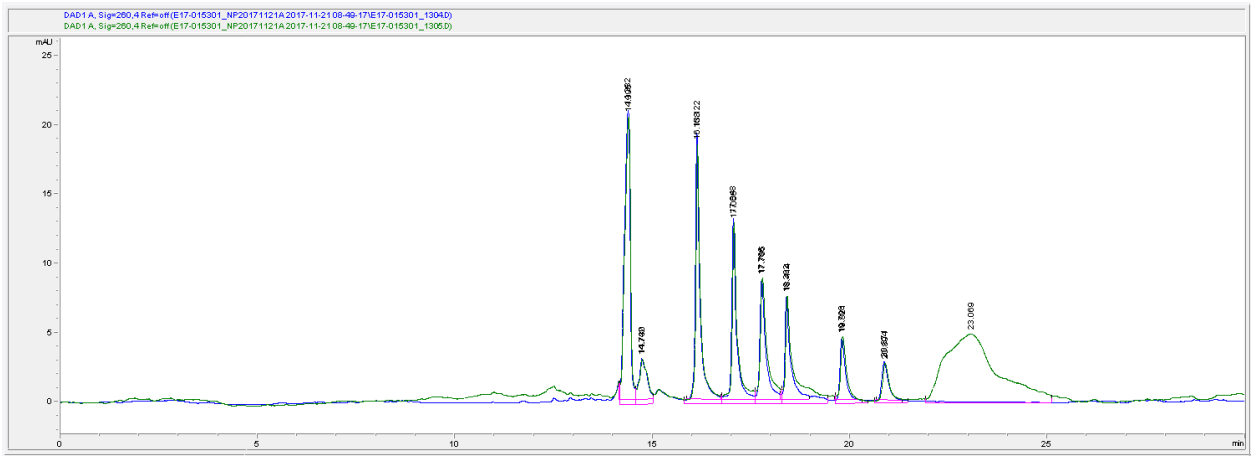

Figure 17. RNA ladder 0.5 mg/ml, heated 80°C 15 min, in sieving medium containing **1.32% HEC (90 kDa) and 1% PVP.** Bare fused silica capillary of ID 50 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar 18 s. Applied voltage: -13 kV. Precoating step with 1% PVP included in method.

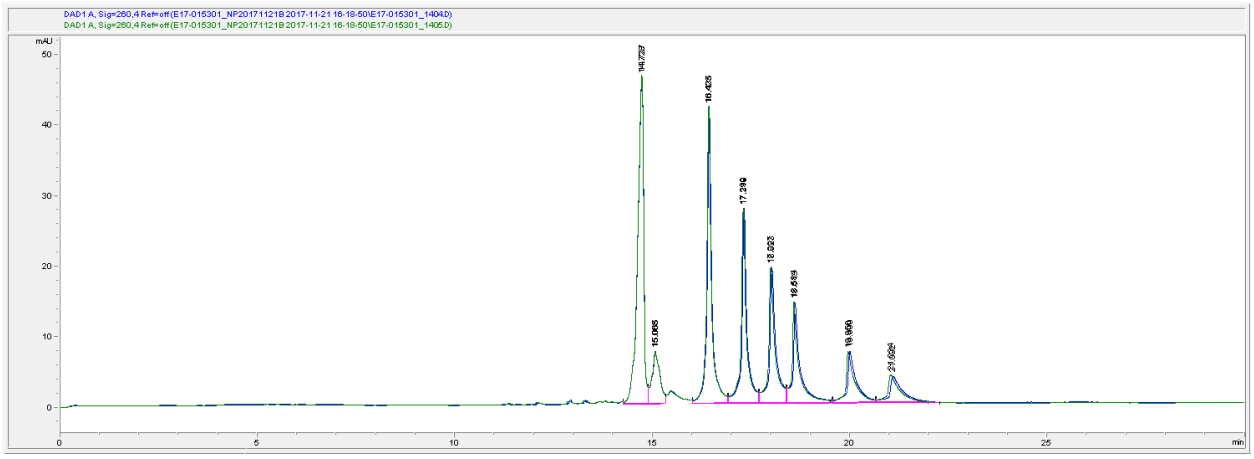

Figure 18. RNA ladder 0.5 mg/ml, heated 80°C 15 min, in sieving medium containing **1.32% HEC (90 kDa)**. Permanently coated capillary of ID 75 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar 30 s. Applied voltage: -13 kV.

Separation of the RNA ladder is improved by a HEC polymer of higher molecular weight such as 250 kDa, *figures 19* and *20*. In the uncoated capillary, the peaks are all baseline separated, uncommon even for separation gel 1 analyses. In the permanently coated capillary

the peaks are baseline separated but just barely, once again a tailing of the peaks can be observed.

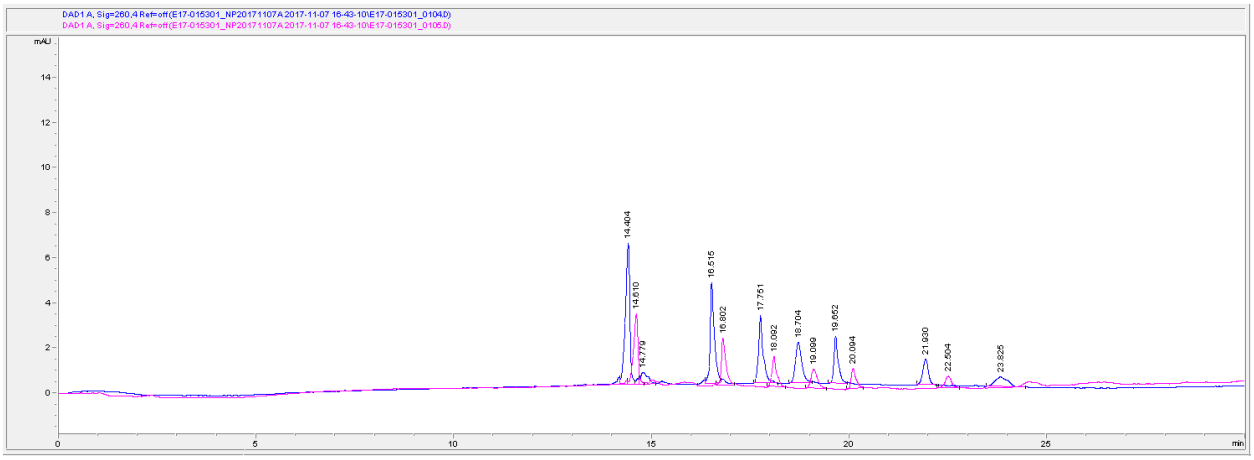

Figure 19. RNA ladder 0.5 mg/ml, heated 80°C 15 min, in sieving medium containing **1.32% HEC (250 kDa)**. **Uncoated capillary** of ID 50 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar 18 s. Precoating step with 1% PVP included in method. Applied voltage: -13 kV.

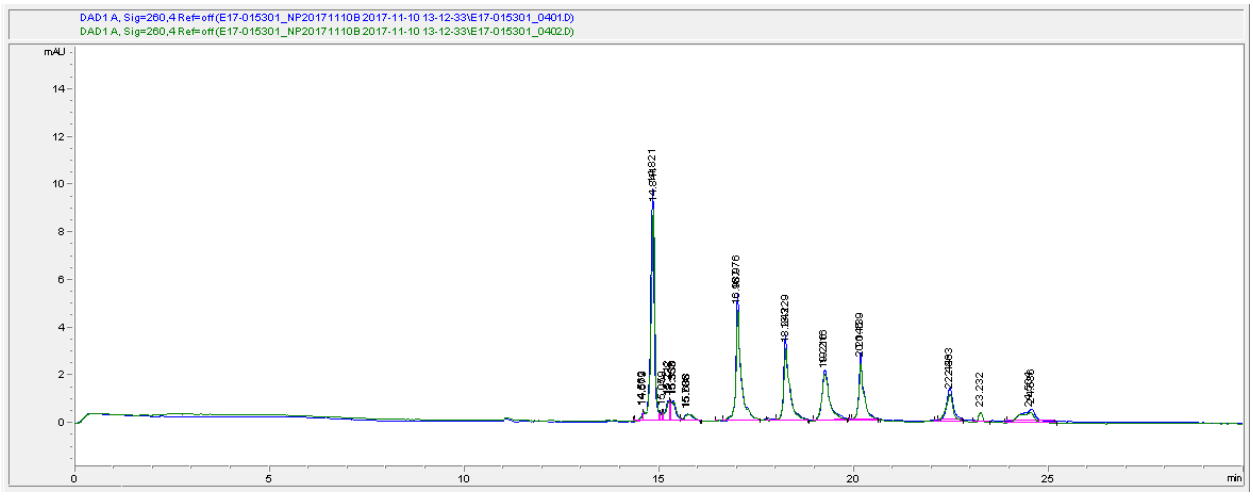

Figure 20. RNA ladder 0.5 mg/ml, heated 80°C 15 min, in sieving medium containing **1.32% HEC (250 kDa). Permanently coated capillary** of ID 75 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar 30 s. Applied voltage: -13 kV.

No results were obtained for HEC 720 kDa as the viscosity of the sieving medium prevented complete matrix refill of the capillary.

It is likely that the separation is improved with the slightly larger 250 kDa HEC compared to that of 90 kDa as the sieving matrix provides a more suitable pore size range for the sample fragments. Further analyses containing HEC (90 kDa) can be seen in *appendix E*.

The permanently coated capillary did not contribute to any significant improvements of the separations and was therefore not further considered due to the added costs and reduced lifetime of these capillaries.

4.3.2.3 PVP/HEC

The initial investigative approach inspired by Boulos *et al*. (Boulos, Cabrices et al. 2008) did not provide any definite result as the viscosity of the sieving medium was too high and could not be successfully filled into the capillary.

Figure 21 shows the separation with a greater share of PVP than HEC (75/25). The separation is very good and there is even baseline separation between peak 4 and 5. As the share of HEC increases, *figures 22* and *23*, the peaks are further separated. However, the intensity also decreases with the increase of HEC. This is consistent with previous experiments, where the viscosity of the sieving medium was increased, either by an increase in polymer concentration or as in this case by a larger portion of polymer with higher viscosity. Higher viscosity leads to more pressure against the sample injection and thereby a lowered injection volume. As previously argued, there is a trade-off to consider between increased separation and intensity of the peaks.

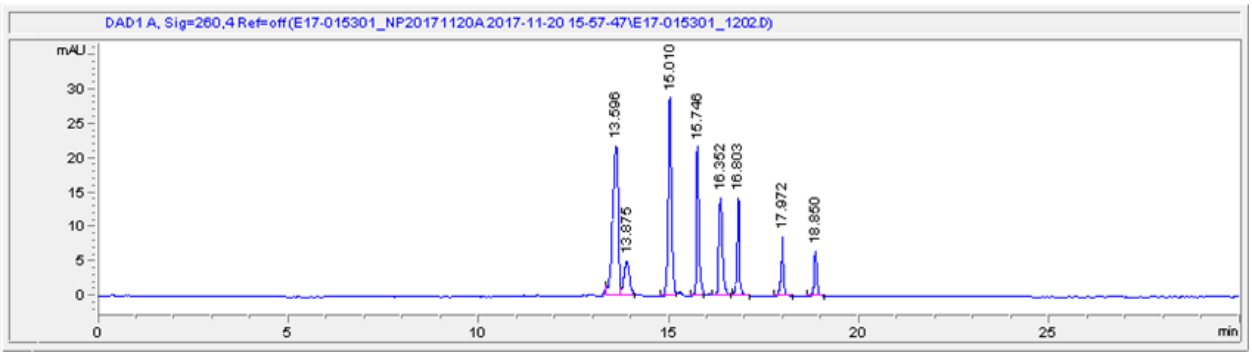

Figure 21. RNA ladder 0.5 mg/ml, heated 80°C 15 min, in sieving medium containing **75/25 PVP (1.3 MDa)/HEC (250 kDa) with a total polymer concentration of 1.32%.** Uncoated capillary of ID 50 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar 18 s. Applied voltage: -13 kV.

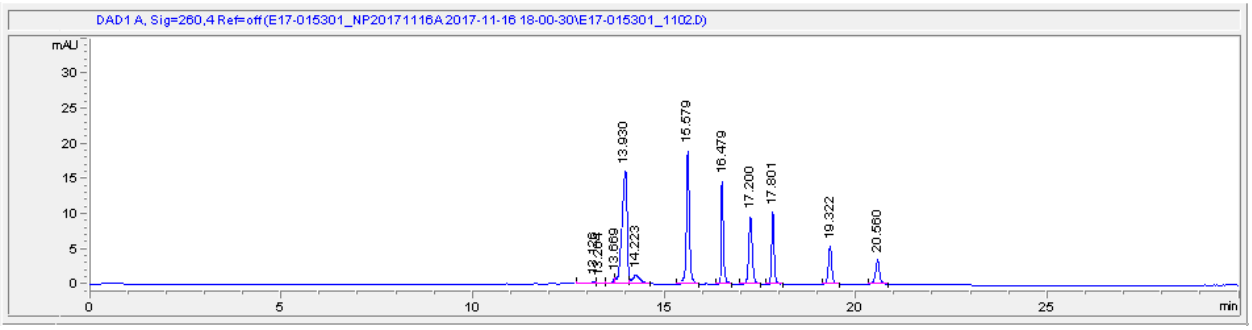

Figure 22. RNA ladder 0.5 mg/ml, heated 80°C 15 min, in sieving medium containing **50/50 PVP (1.3 MDa)/HEC (250 kDa) with a total polymer concentration of 1.32%.** Uncoated capillary of ID 50 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar 18 s. Applied voltage: -13 kV.

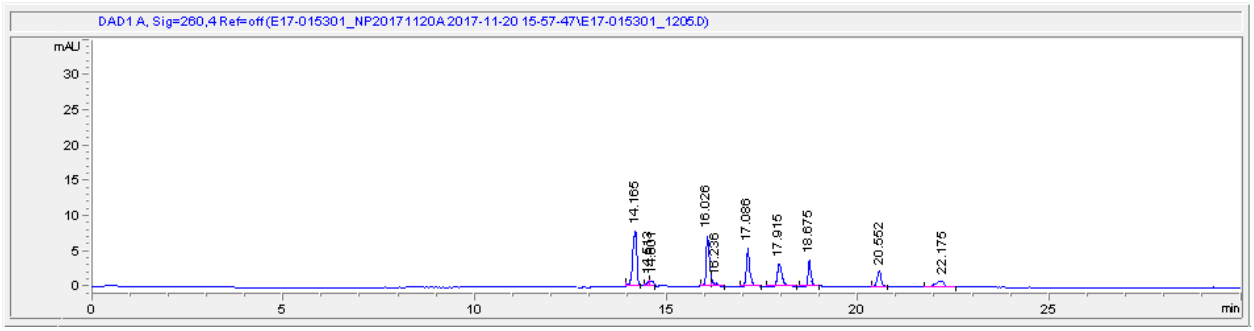

Figure 23. RNA ladder 0.5 mg/ml, heated 80°C 15 min, in sieving medium containing **25/75 PVP (1.3 MDa)/HEC (250 kDa) with a total polymer concentration of 1.32%.** Uncoated capillary of ID 50 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar 18 s. Applied voltage: -13 kV.

Additional investigation of the effect of polymer composition was carried out. The concentration of PVP (1.3 MDa) was set to 1.32% with an increasing added concentration of HEC (250 kDa) The idea was that the amount of PVP would enable suppression of EOF and the addition of HEC would improve the separation. *Figure 24* and *25* confirm that the separation is enhanced by addition of a higher amount of HEC. However, the addition of 1.5% HEC resulted in a too high viscosity to efficiently fill the capillary and no results were obtained. As can be seen in *figure 25* a mixture of PVP and HEC at a total polymer concentration of 2.32% has a detrimental effect on the baseline. This could potentially have a negative effect on the detection of fragments due to the evident drop in peak intensity.

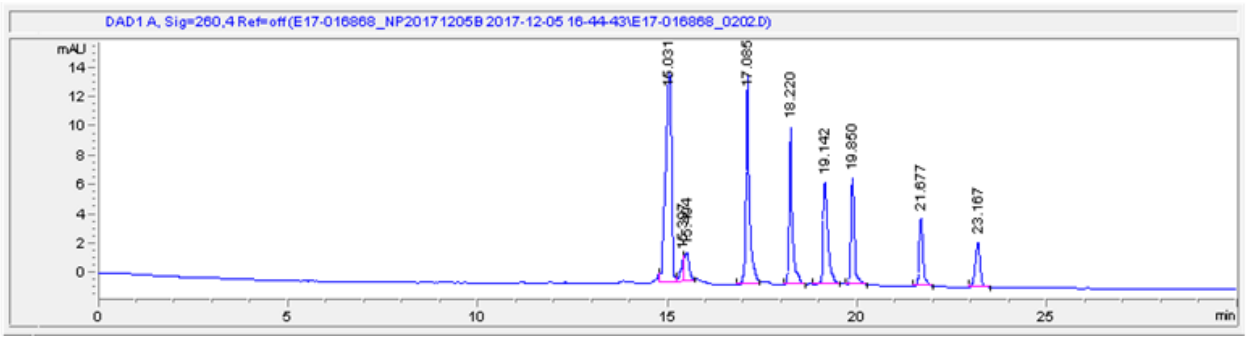

Figure 24. RNA ladder 0.5 mg/ml, heated 80°C 15 min, in sieving medium containing **1.32% PVP + 0.5% HEC (250 kDa).** Uncoated capillary of ID 50 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar 18 s. Applied voltage: -13 kV.

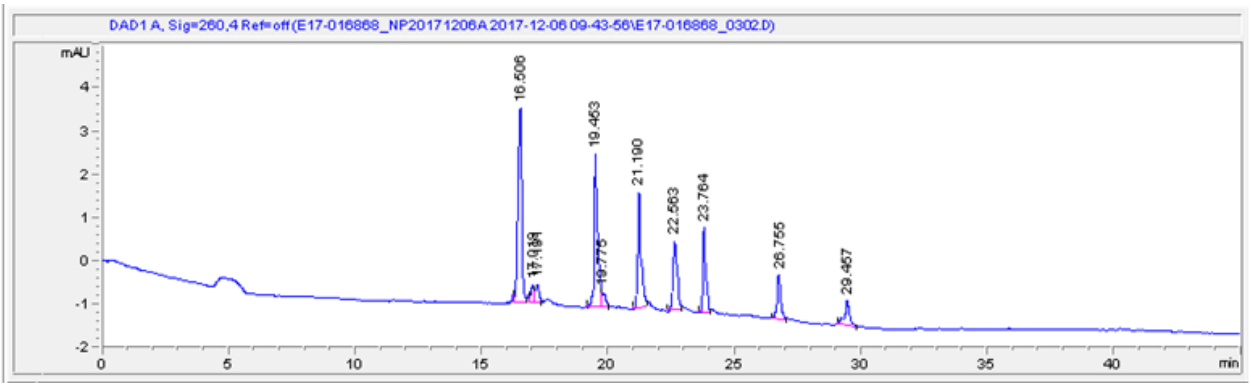

Figure 25. RNA ladder 0.5 mg/ml, heated 80°C 15 min, in sieving medium containing **1.32% PVP + 1.0% HEC (250 kDa).** Uncoated capillary of ID 50 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar 18 s. Applied voltage: -13 kV.

4.3.2.4 Resolution

Table 6 contains the resolutions for the RNA ladder peaks (*equations 2* and *3*) of each investigated sieving medium. The numbers reveal that the 1.32% PVP + 1.0% HEC sieving medium gave the highest resolution between almost all peaks.

Table 6. Resolution data for each alternative sieving medium. T = migration time and the number specifies the peak (1 = 100 nt, 2 = 200nt, 3 = 300 nt, 4 = 400 nt, 5 = 500 nt, 6 = 750 nt, 7 = 1000 nt).

Sieving medium	R 1-2	R 2-3	R 3-4	R 4-5	R 5-6	R 6-7
25/75 PVP/HEC (250 kDa), tot 1.32%	9,88	6,87	4,28	3,98	11,3	0,649
50/50 PVP/HEC (250 kDa), tot 1.32%	8,88	6,99	4,99	4,26	11,3	0,495
75/25 PVP/HEC (250 kDa), tot 1.32%	7,31	5,51	4,07	3,01	8,50	0,351
1.32% PVP + 0.5% HEC (250 kDa)	9,65	6,66	4,75	3,67	10,6	0,596
1.32% PVP + 1.0% HEC (250 kDa)	11,9	8,02	5,45	4,98	12,7	1,08
1.32% PVP (360 kDa)	5,74	4,23	3,13	2,11	5,15	0,231
2% PVP (360 kDa)	7,99	5,88	4,02	2,67	6,92	0,383
2% PVP (1.3 MDa)	7,88	5,66	3,83	2,52	6,25	0,402
5% PVP (1.3 MDa)	10,4	7,09	3,54	2,07	3,73	1,66

4.3.3 Additives in sieving medium

The following subsection describes the result of adding different chemicals to the sieving medium in an attempt to achieve improved separation by altering the conformation of the sample and/or the polymer entanglement. By achieving a beneficial conformation of the sample through the sieving medium and process of separation the sample itself can be applied untreated. This simplifies the preparation process of the analysis and is beneficial for the reproducibility of the analysis.

4.3.3.1 Magnesium

Figure 26 and *27* reveal the result of separation with addition of 2 mM $MgCl_2$ in the sieving medium. According to the literature (Draper 2004, Mohamadi, Kaji et al. 2008), Mg^{2+} can binding both to the RNA molecule and the PVP polymer and stabilize certain conformations. Studying the separation of the EPO mRNA sample in *figure 26* the post-main peaks previously eliminated by heating the sample at 80°C for 15 min have reappeared. This indicates an interaction with the Mg^{2+}, resulting in alternative secondary structures or even multimers. Also, the RNA ladder separation indicate alternative conformations for the fragments in the sample, see *figure 27*. However, this results in a poor separation with peak splits. Overall, the addition of $MgCl_2$ does not seem to have a positive effect.

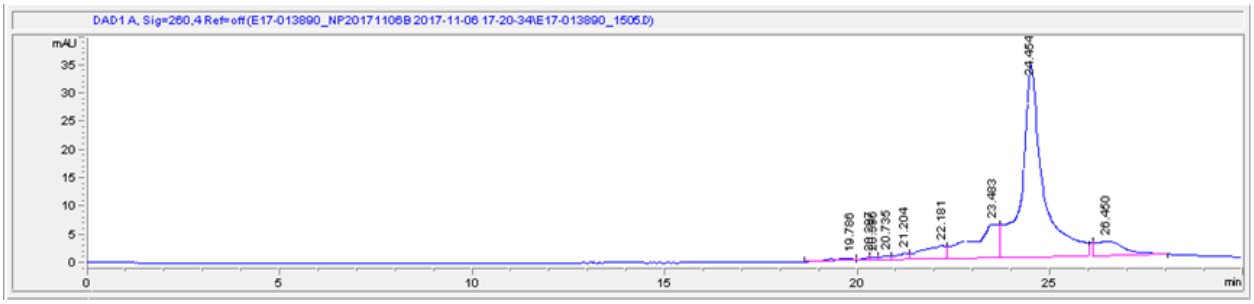

Figure 26. EPO mRNA 0.5 mg/ml, heated 80°C 15 min, in **separation gel 1 + 2 mM $MgCl_2$**. Uncoated capillary of ID 50 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar 18 s. Applied voltage: -13 kV.

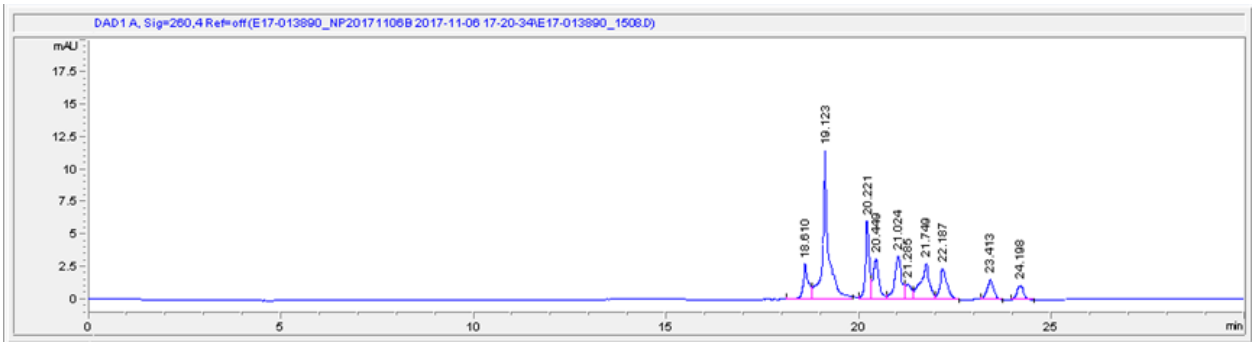

Figure 27. RNA ladder 0.5 mg/ml, heated 80°C 15 min, in **separation gel 1 + 2 mM $MgCl_2$**. Uncoated capillary of ID 50 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar 18 s. Applied voltage: -13 kV.

4.3.3.2 Acetonitrile

Using acetonitrile as sieving medium additive does not show any indication of being beneficial for CGE separation of RNA molecules. The EPO mRNA separation in *figure 28* shows a slight increase in post-main peaks and is otherwise not visually different from an analysis without acetonitrile. *Figure 29* with the separation of mRNA however reveal a negative effect on the separation as the peaks are wider and less separated than usual.

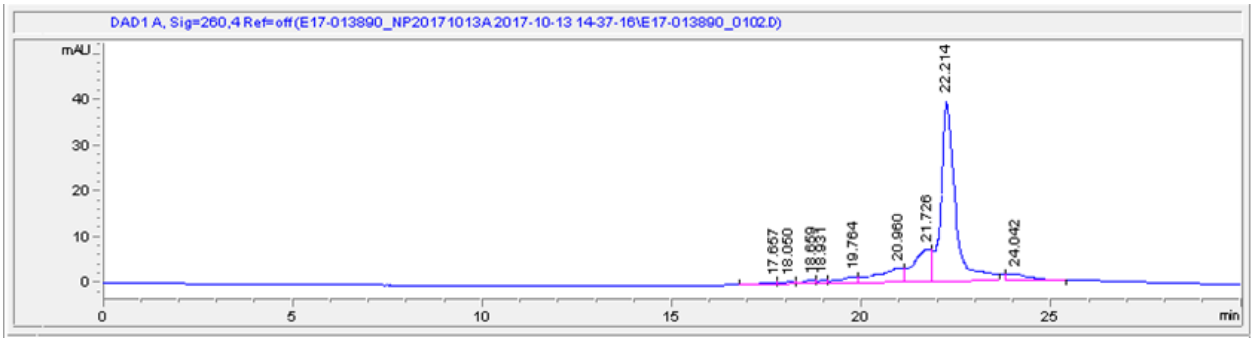

Figure 28. EPO mRNA 0.5 mg/ml, heated 80°C 15 min, in **separation gel 1 with 20% acetonitrile**. Uncoated capillary of ID 50 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar 18 s. Applied voltage: -13 kV.

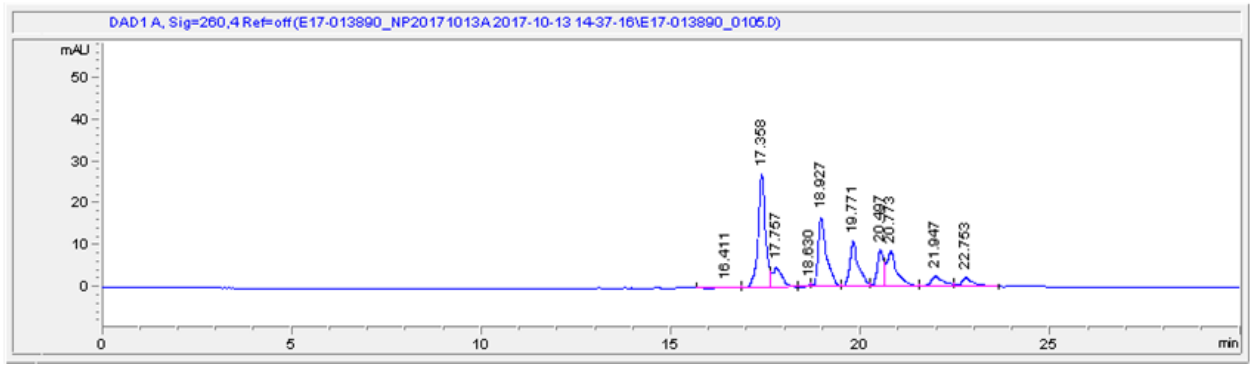

Figure 29. RNA ladder 0.5 mg/ml, heated 80°C 15 min, in **separation gel 1 with 20% acetonitrile**. Uncoated capillary of ID 50 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar 18 s. Applied voltage: -13 kV.

4.3.3.3 Sucrose

Sucrose was explored as an additive on an intuition that it would perform as a viscosity modifier in the sieving medium. The initial test of adding sucrose to a percentage of 10% of the total solution gave a high viscosity which hindered a successful separation analysis. Nevertheless, by excluding the glycerol the viscosity was at a manageable level and could easily be applied to the CGE system. Surprisingly, the replacement of glycerol with 10% sucrose proved to have a positive effect on separation. The separations obtained from the experiment, *figure 30* and *31*, are very similar to those obtained from the original separation gel 1, *figure 12* and *13*. However, looking closer at the RNA ladder electropherogram in *figure 31* the separation of peak number 4 and 5 is in this case good. Additionally, the peaks have a relatively high intensity, and are sharp without any visible tailing.

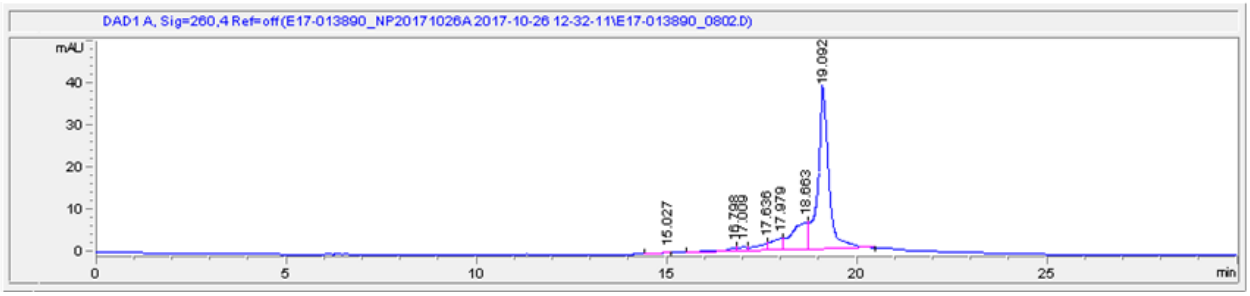

Figure 30. EPO mRNA 0.5 mg/ml, heated 80°C 15 min, in **separation gel 1 with 10% glycerol replaced with sucrose**. Uncoated capillary of ID 50 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar 18 s. Applied voltage: -13 kV.

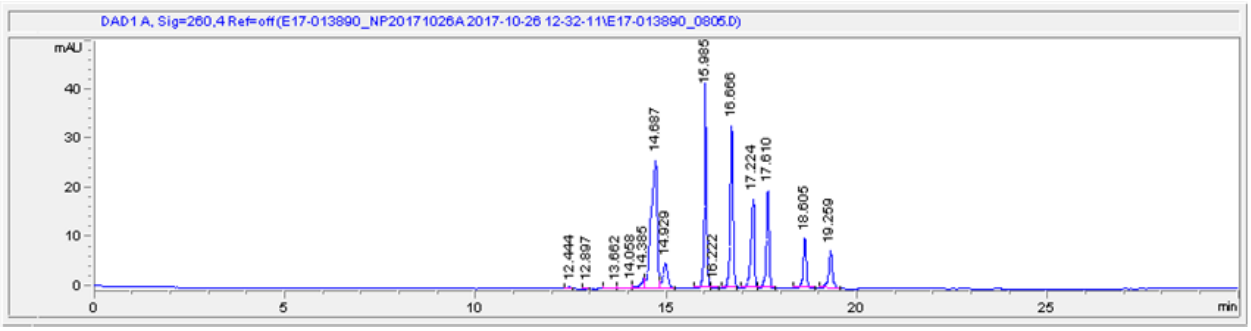

Figure 31. RNA ladder 0.5 mg/ml, heated 80°C 15 min, in **separation gel 1 with 10% glycerol replaced with sucrose**. Uncoated capillary of ID 50 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar 18 s. Applied voltage: -13 kV.

4.3.3.4 Formaldehyde

Formaldehyde as an additive in the sieving medium did not lead to an improved RNA separation. The resulting electropherograms from the experiment, *figure 32* and *33*, reveal bulky peaks and a suspected degradation of the first peak in the RNA ladder separation.

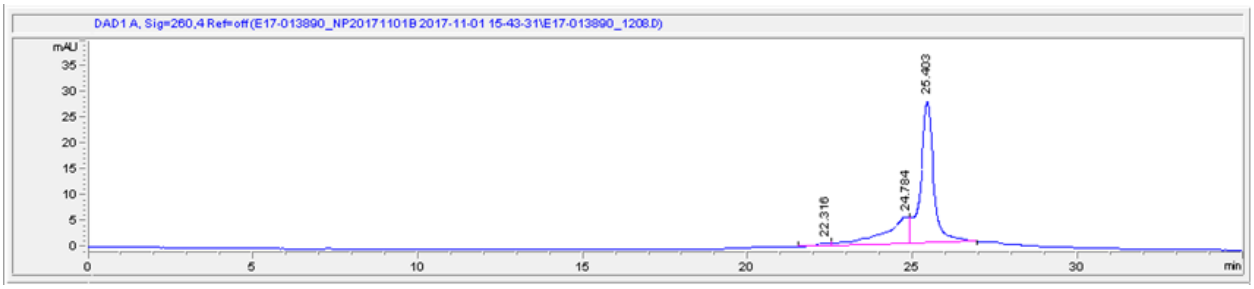

Figure 32. EPO mRNA 0.5 mg/ml, heated 80°C 15 min, in **separation gel 1 with 20% formaldehyde**. Uncoated capillary of ID 50 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar 18 s. Applied voltage: -13 kV.

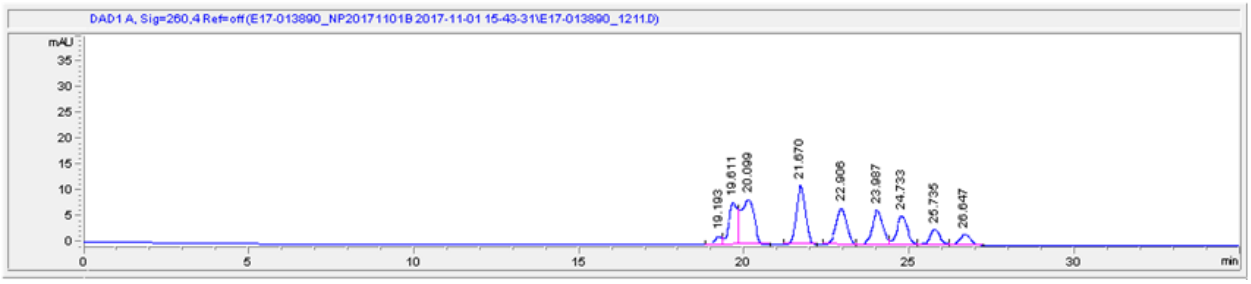

Figure 33. RNA ladder 0.5 mg/ml, heated 80°C 15 min, in **separation gel 1 with 20% formaldehyde**. Uncoated capillary of ID 50 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar 18 s. Applied voltage: -13 kV.

4.3.3.5 Acetic acid

Despite the very promising results presented in the article by Sumitomo *et al.* (2009) the results of the acetic acid experiments show no improvement on the separation of the RNA samples. In fact, for both the EPO mRNA and the RNA ladder the separation is very poor, see *figure 34* and *35*. The poor separation is likely a result of the low pH of the sieving medium due to the added acetic acid. While all other separations are carried out at a pH around 7.5 the measured pH of the 2 M acetic acid sieving medium is at 3. This likely causes degradation of the RNA samples or the sieving media and also the suppression of EOF mentioned in *"2.2.2 Electroosmotic flow"* and *"2.2.4 Capillaries"*. It is also possible that the low pH affects the charge of the RNA and thereby its migration through the sieving medium.

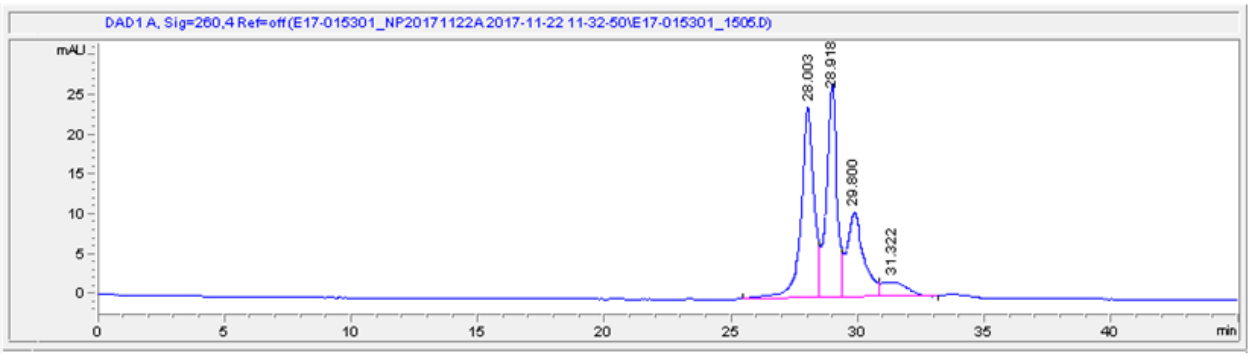

Figure 34. RNA ladder 0.5 mg/ml, heated 80°C 15 min, in **separation gel 1 + 2 M acetic acid**. Uncoated capillary of ID 50 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar 18 s. Applied voltage: -13 kV.

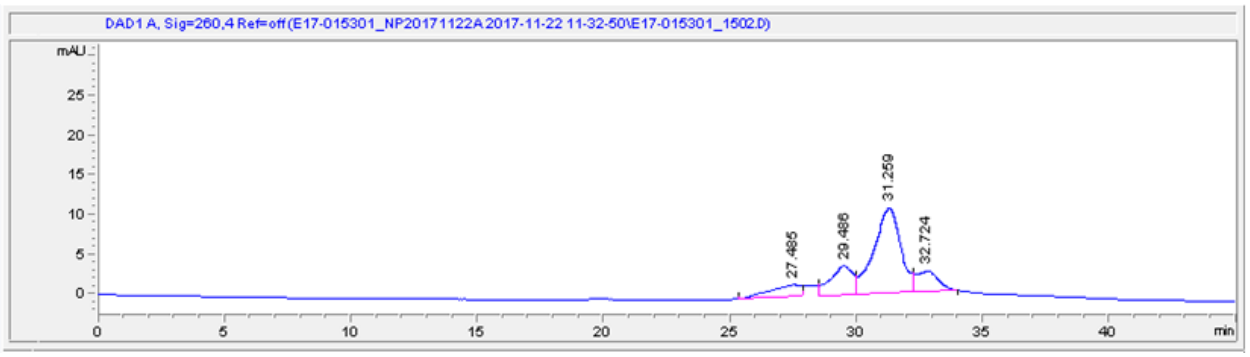

Figure 35. EPO mRNA 0.5 mg/ml, heated 80°C 15 min, in **separation gel 1 + 2 M acetic acid**. Uncoated capillary of ID 50 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar 18 s. Applied voltage: -13 kV.

4.3.3.6 Urea

The addition of urea to a final concentration of approximately 3.3 M in the sieving medium, *figures 36* and *37*, give significantly different results compared to approximately 3.2 M urea as sample additive (*figures 10* and *11*). Urea used as a sample additive resulted in sharper peaks, especially for the EPO mRNA sample seen in *figure 11*, and thereby also further enabling the characterisation of content in the pre-main peak. In the case of urea as sieving medium additive the peaks are instead broader and resolution between most peaks visibly poorer. These seemingly diverse results are likely due to the urea's different ability of integrating with the RNA molecules in the different settings. While simply adding urea to the sieving medium has an effect on the viscosity of the solution, the binding to the biomolecules does not seem to be efficient enough to give any beneficial conformational change to enhance the separation. When instead studied as a potential sample additive these conditions likely provided the urea opportunity to bind to the RNA.

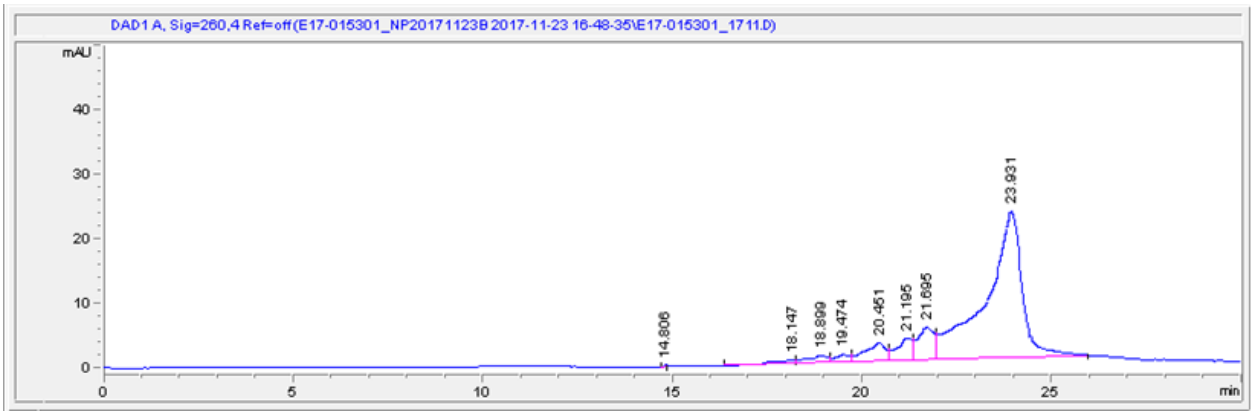

Figure 36. EPO mRNA 0.5 mg/ml, heated 80°C 15 min, in **separation gel 1 + 3.3 M urea**. Uncoated capillary of ID 50 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar 18 s. Applied voltage: -13 kV.

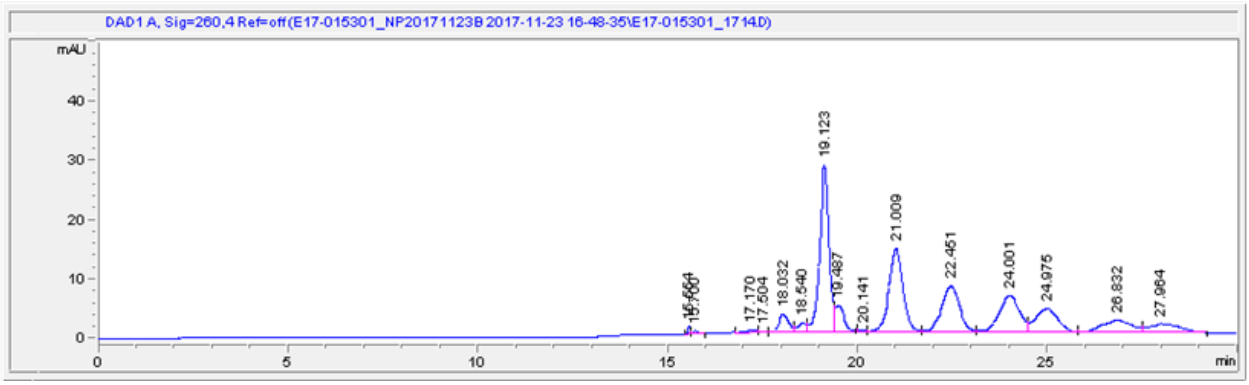

Figure 37. RNA ladder 0.5 mg/ml, heated 80°C 15 min, in **separation gel 1 + 3.3 M urea**. Uncoated capillary of ID 50 µm, effective length 40 cm at 25°C. Injection parameters 80 mbar 18 s. Applied voltage: -13 kV.

4.3.3.7 Resolution

Comparing the results of the sieving media-tests, *table 7*, only one additive gives indication of enhancing the separation i.e. the substitution of glycerol to sucrose. This is a somewhat

surprising result as the other chemicals tested were derived from successful trials described in literature, while sucrose was tested on a whim for its viscosity influencing qualities.

Table 7. Results from resolution calculations of sieving medium with additives. T = migration time and the number specifies the peak (1 = 100 nt, 2 = 200nt, 3 = 300 nt, 4 = 400 nt, 5 = 500 nt, 6 = 750 nt, 7 = 1000 nt).

Additives	R 1-2	R 2-3	R 3-4	R 4-5	R 5-6	R 6-7
2 mM $MgCl_2$	4,95	3,25	2,19	1,28	3,79	0,314
20% acetonitrile	4,54	2,26	2,11	0,75	2,28	0,322
10% sucrose (-glycerol)	5,78	5,34	3,62	2,47	7,16	0,262
20% formaldehyde	4,06	2,17	1,77	1,26	1,69	0,37
2 M acetic acid	1,179	0,968	-	-	-	-
3.3 M urea	3,331	1,814	1,650	0,948	1,584	0,453

Overall, the sieving medium additives did not live up to expectations. Most of them not only did not improve separation but effectively contaminated the separation set up, causing problems for reproducibility and life span of the capillaries.

4.4 Test of optimised CGE separation parameters

Initial results from gathering all the beneficial components found during the project into one optimised method were not satisfactory. Hardly any peaks could be detected and the unstable baseline and current indicated a faulty matrix refill into the capillary (data not shown). To decrease the viscosity of the sieving medium and thereby enabling a complete capillary refill the temperature of the cassette was raised to 60°C. This resulted in peaks that were well separated (see *figures A21* and *A22* in *appendix F*). However, the instability of the baseline was still an issue. Decreasing the temperature to 40°C and changing the injection parameters to 80 mbar for 30 s lead to improved baseline stability, while the peaks were still detectable despite the increase in viscosity (see *figures A23-A25* in *appendix F*). Nevertheless, it could be established that the concentration of HEC was part of the problem with the instability of the baseline as these tendencies could be traced back to the initial test of 1.32% PVP + 1.0% HEC (*figure 25*). A new sieving medium of 1.32% PVP (1.3 MDa) + 0.5% HEC (250 kDa), 10% sucrose, 15% HEPES pH 7.5 and RNase free water was prepared and analysis was

performed at similar conditions as the previous run (40°C, 80 mbar 30 s injection). This improved the separation further. Another experiment was performed for investigation of further improvements. The capillary was substituted to a shorter one with ID 75 µm and effective length 72 cm to further simplify sieving medium refill and stabilising the baseline. The final results are seen in *figures 38* and *39*. Evidently, these results are superior compared previous analyses (such as those with separation gel 1 and FACE sieving medium seen in *figure 12* and *13*) of the same samples as there is baseline separation between all peaks, the intensity of the peaks is good and the baseline is stable.

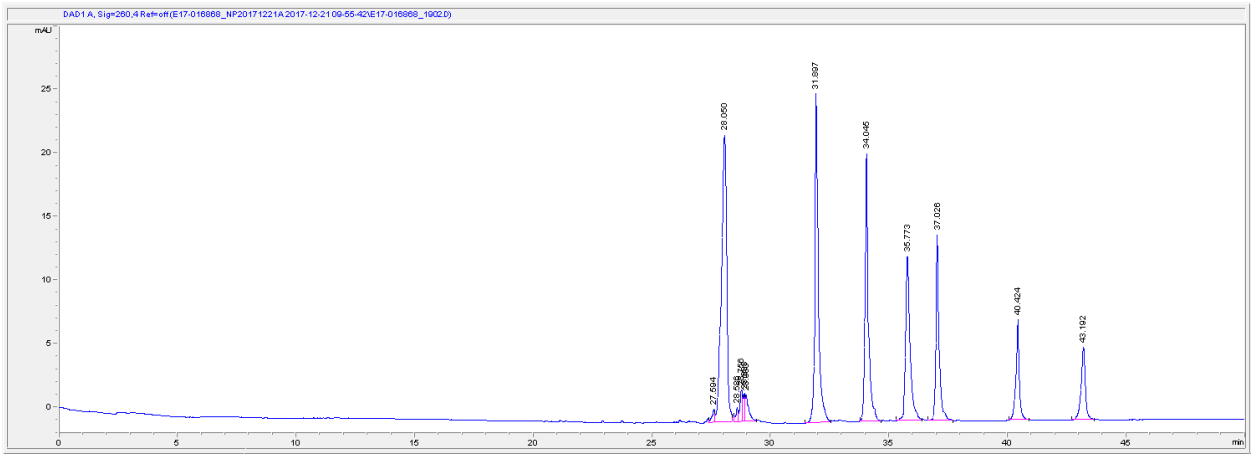

Figure 38. RNA ladder 0.5 mg/ml in **optimised sieving medium 2.0 (1.32% PVP + 0.5% HEC, 10% sucrose, 15% HEPES pH 7.5, RNase free water)** in ID 75 µm, effective length 72 cm capillary at 25°C. Injection parameters 80 mbar, 30 s. Applied voltage: -21 kV.

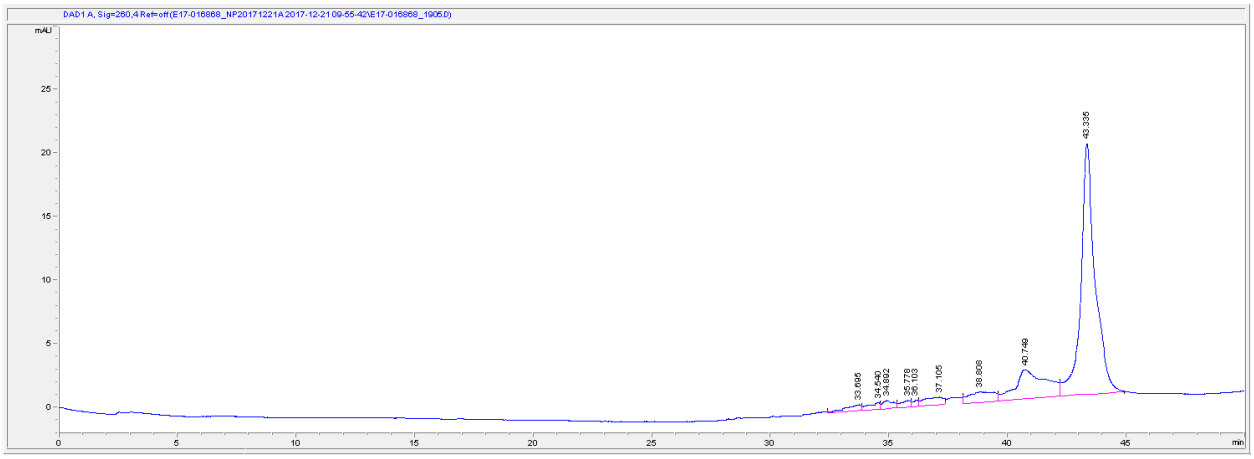

Figure 39. EPO mRNA 0.5 mg/ml in **optimised sieving medium 2.0 (1.32% PVP + 0.5% HEC, 10% sucrose, 15% HEPES pH 7.5, RNase free water)** in ID 75 µm, effective length 72 cm capillary at 25°C. Injection parameters 80 mbar, 30 s. Applied voltage: -21 kV.

Additionally, an EPO mRNA sample with 3.2 M urea as additive was analysed using the same optimised method and conditions, *figure 40*. This addition enhanced the separation of shortmers in the sample further as they visually appeared sharper and thereby more distinguishable.

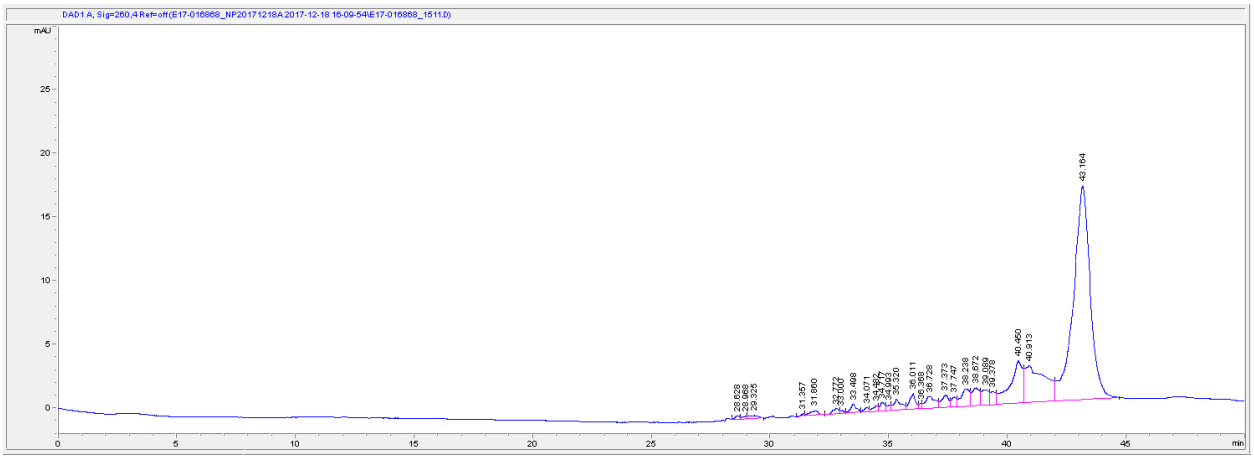

Figure 40. EPO mRNA 0.5 mg/ml **+ 3.2 M urea** in **optimised sieving medium 2.0 (1.32% PVP + 0.5% HEC, 10% sucrose, 15% HEPES pH 7.5, RNase free water)** in ID 75 µm, effective length 72 cm capillary at 25°C. Injection parameters 80 mbar, 30 s. Applied voltage: -21 kV.

In total, the final optimised method for separation of the mRNA sample included an instrumental set up with a capillary of ID 75 µm and effective length 72 cm at 25°C and use of a heavy wash for capillary regeneration, a sample treatment of heating at 80°C for 15 min together with 3.2 M urea as additive and a sieving medium composition of 1.32% PVP + 0.5% HEC with sucrose as viscosity modifier.

A table containing the peak resolutions of all investigated optimal sieving media can be seen in *appendix G*.

5 Conclusion

The aim of this project was to optimise the separation of shortmers in RNA samples analysed by CGE. There is a great number of factors that influence the separation quality of a CGE analysis. The key factors are the instrumental parameters, composition of the sieving medium and treatment of the sample. Through the course of the project many alternative experimental set ups have been explored and comparatively evaluated to a commercially available and most recent from literature sieving medium used for similar analyses.

Initial comparative studies on instrumental parameters indicated that a capillary with wider ID and longer effective length would be beneficial for the analysis. The method parameters, voltage/cm and injection volume, were set individually for each capillary dimension to create a fair comparative setting. However, there is still a question of whether a method optimisation for each individual capillary dimension would enable enhancement of the results. For example, the longest capillaries used were provided with the maximum voltage set by the instrument, while the shorter capillaries were adjusted to the same volt/cm with a lower applied voltage and not to operate at their full capacity. Using the same voltage as applied for the longest capillary would give a faster and possibly better separation than shown in these

adjusted experiments. Also, the cassette temperature was examined to give an indication of what temperature was optimal during separation. The result from this did not provide a clear-cut optimal temperature. Yet, indication of the diverse effects of the different temperatures which could all individually be advantageous for different analysis conditions could be observed. For instance, a higher temperature allowed for faster separation and higher intensity of the peaks while separation at lower temperatures enabled detection of additional peaks. However, further investigation with a constant current could provide more insight into the effect of cassette temperature on the separation. In addition, a capillary heavy wash procedure pre-sieving medium refill was established to fully discard any residual medium from the previous run. This significantly improved reproducibility of repeated runs.

Investigation of sample treatment methods were carried out with the goal to find an optimal conformation of RNA molecules for CGE separation. It is assumed that elimination of transient secondary structures offers better reproducibility and an intensity increase of the main peak. When the sample was heated at 80°C for different lengths of time it was found that heating for 15 minutes resulted in disappearance of the post-main peaks without significant degradation of the main peak. This pre-treatment method was then used consistently in all sample preparations throughout the project. The effect of chemical additives in sample for the conformational stability of RNA was also examined. Addition of $MgCl_2$ caused contamination difficulties in the entire CGE set-up. The addition of urea before heating however, demonstrated a potential positive effect by making the peak shape of the pre-main peaks more sharp and distinguishable.

Addition of chemical additives to the sieving medium was also examined after a literature study. It was hypothesized that this would allow analysis of an unaltered sample with the denaturing effects in the separation medium. However, this approach only rarely led to an improvement in the separation. Instead, it was often difficult to regenerate the capillary. Surprisingly, the only additive that had a positive effect on resolution was sucrose. By exchanging the viscosity modifier 10% glycerol of separation gel 1 to the same concentration of sucrose separation was improved. Compared to the other chemicals tested, sucrose did not have any detrimental effect on the regeneration of the system.

After an extensive literature search the investigation of alternative sieving medium compositions lead to a primary study of two specific polymers, i.e. PVP and HEC. Both have different beneficial qualities, PVP with its self-coating ability and low viscosity and HEC with its established sieving ability, and they are widely commercially available. Both polymers were investigated with different molecular weights and at different concentrations. Sieving medium containing a mix of the two polymers at different proportions and total concentrations showed promising separation results and resolution calculations confirmed this improvement. Keeping the PVP percentage constant for basic separation and EOF suppression, the separation capacity seemed to increase with increasing percentage of HEC added. However, this also had a grave impact on the viscosity making the capillary replenishment severely more difficult.

Besides an appreciative visual evaluation of the separations obtained, the difference in separation capabilities were also evaluated by peak resolutions. However, the peak resolution data does not reveal the complete performance of a separation. Baseline stability, peak intensity and shape, ease of capillary refill and capillary regeneration are some of the important qualities to consider in addition to peak resolution data.

Initial application of all separation improving parameters into one analysis method proved not to be optimal for CGE analysis. By receding some of the factors to a slightly lesser separation capability the method gained stability, reproducibility and increased time efficiency. The final analysis set up involved RNA samples heated for 80°C for 15 min, analysed with a capillary of 75 µm ID and 72 cm effective length at 25°C. The sieving medium consisting of mixed polymers (1.32% PVP (1.3 MDa) + 0.5% HEC (250 kDa)) and 10% sucrose as viscosity modifier and the method included the newly established capillary heavy wash procedure. Addition of approximately 3.2 M urea to the EPO mRNA sample before heating was also applied for improved peak shape. Though the resolution of the separation has been greatly improved within the relevant fragment size range there is still room for further optimisation.

5.1 Future work and prospects

This project has shown the potential of using mixed polymer sieving media for enhanced separation performance in CGE. To further optimise the developed sieving medium a design study using multi-objective optimization (MOO) predictions for optimal composition should be applied similarly to the work of De Scheerder *et al.* (De Scheerder, Sparén et al. submitted). Moreover, the method parameters, such as volt/cm and injection volume, should also be optimised specifically for the capillary and medium of choice. Validation tests and degradation studies of mRNA should then be performed to establish the robustness, reproducibility, specificity and stability indicating power of the method. Also, the sample pre-treatment with urea and heating should be further explored for optimal concentration of the additive and ideal time for heating. Furthermore, in future method optimisation studies additional polymers and modified polymers as well as other modes of CE could be explored to find beneficial combinations for specific size range separations.

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Appendix A

Capillary dimensions

Figure A1 – A6 shows all the electropherograms from the capillary dimension investigation in "4.1.1 Capillary dimensions".

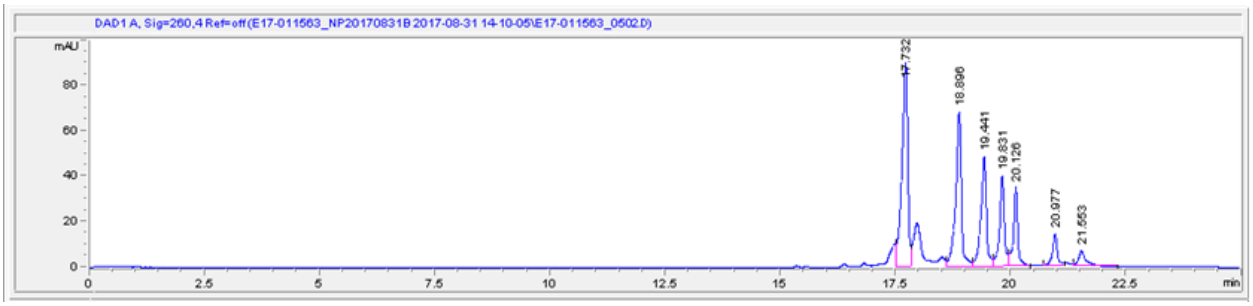

Figure A1. RNA ladder 1 mg/ml, heated 80°C for 2 min, in FACE sieving medium in **ID 50 µm, effective length 40 cm** capillary at 25°C. Injection parameters 80 mbar, 17 s. Applied voltage: -12 kV.

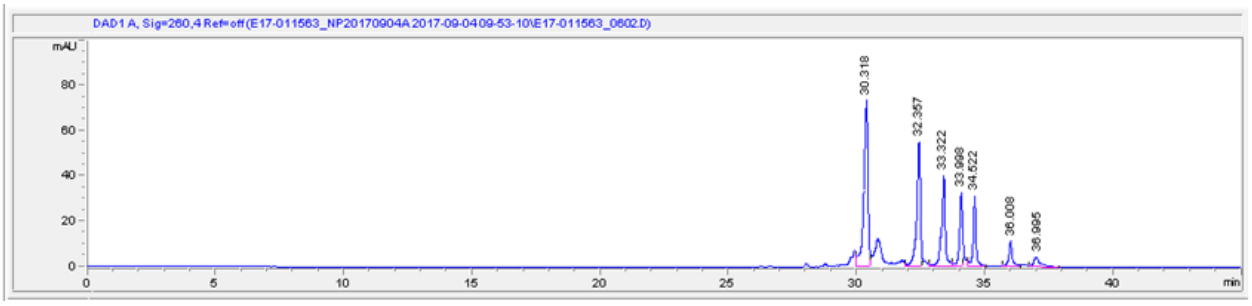

Figure A2. RNA ladder 1 mg/ml, heated 80°C for 2 min, in FACE sieving medium in **ID 50 µm, effective length 72 cm** capillary at 25°C. Injection parameters 80 mbar, 30 s. Applied voltage: -21 kV.

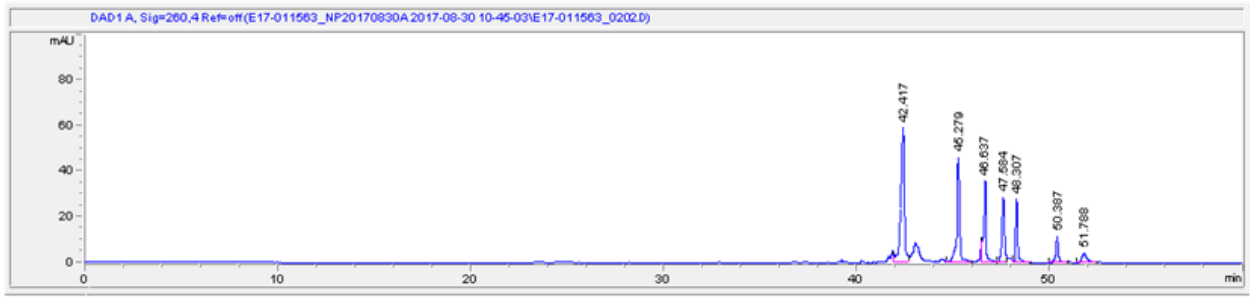

Figure A3. RNA ladder 1 mg/ml, heated 80°C for 2 min, in FACE sieving medium in **ID 50 µm, effective length 104 cm** capillary at 25°C. Injection parameters 80 mbar, 43 s. Applied voltage: -30 kV.

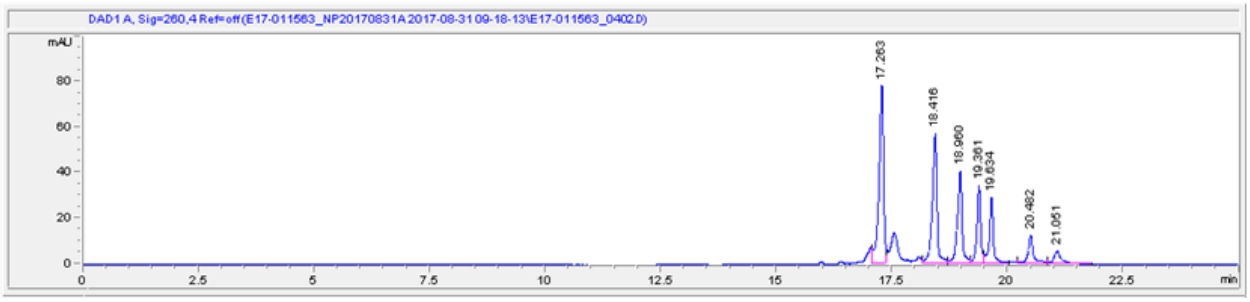

Figure A4. RNA ladder 1 mg/ml, heated 80°C for 2 min, in FACE sieving medium in **ID 75 µm, effective length 40 cm** capillary at 25°C. Injection parameters 30 mbar, 9 s. Applied voltage: -12 kV.

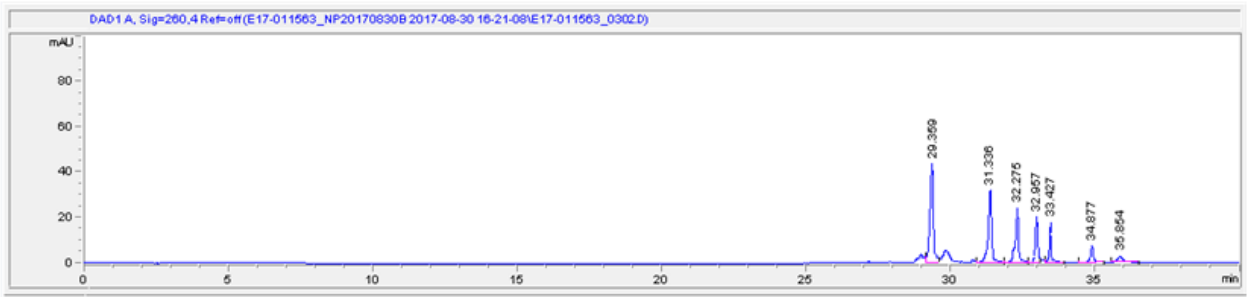

Figure A5. RNA ladder 1 mg/ml, heated 80°C for 2 min, in FACE sieving medium in **ID 75 µm, effective length 72 cm** capillary at 25°C. Injection parameters 30 mbar, 16 s. Applied voltage: -21 kV.

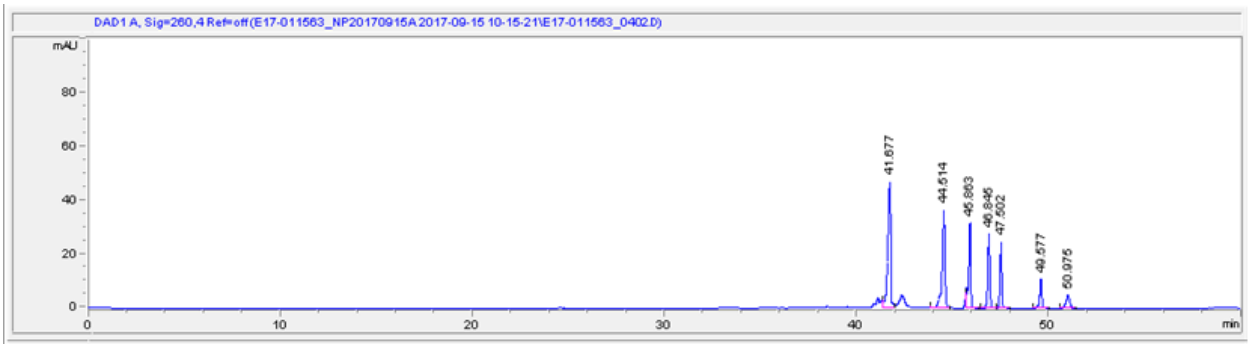

Figure A6. RNA ladder 1 mg/ml, heated 80°C for 2 min, in FACE sieving medium in **ID 75 µm, effective length 104 cm** capillary at 25°C. Injection parameters 30 mbar, 23 s. Applied voltage: -30 kV.

Appendix B

Precoating and prewash evaluation

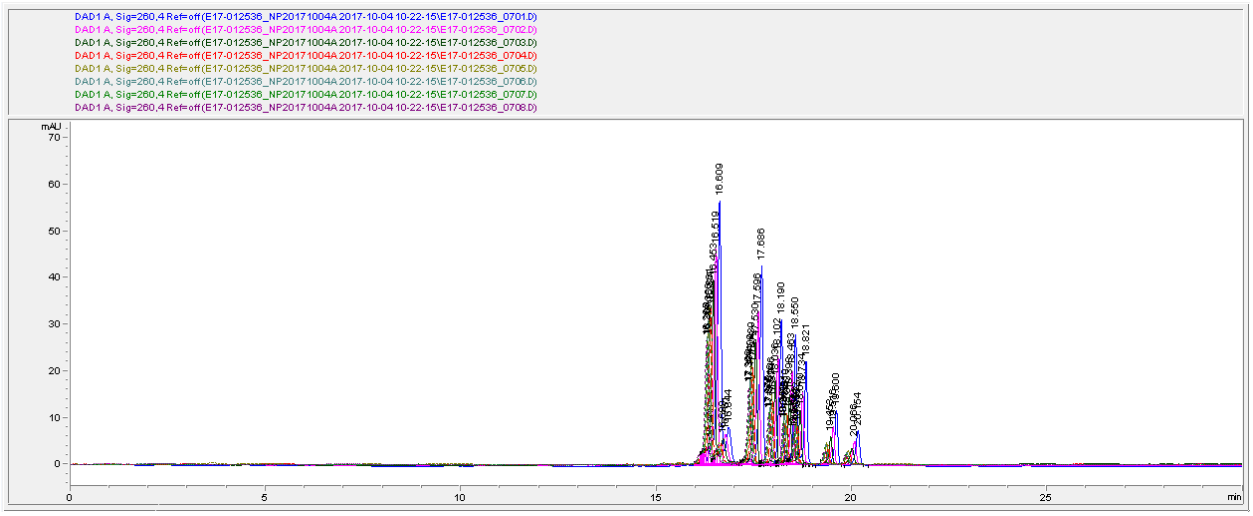

Figure A7. **Method including 1% PVP precoating step**. RNA ladder 0.5 mg/ml, heated 80°C for 15 min, in FACE sieving medium in ID 50 µm, effective length 40 cm capillary at 25°C. Injection parameters 80 mbar, 18 s. Applied voltage: -13 kV.

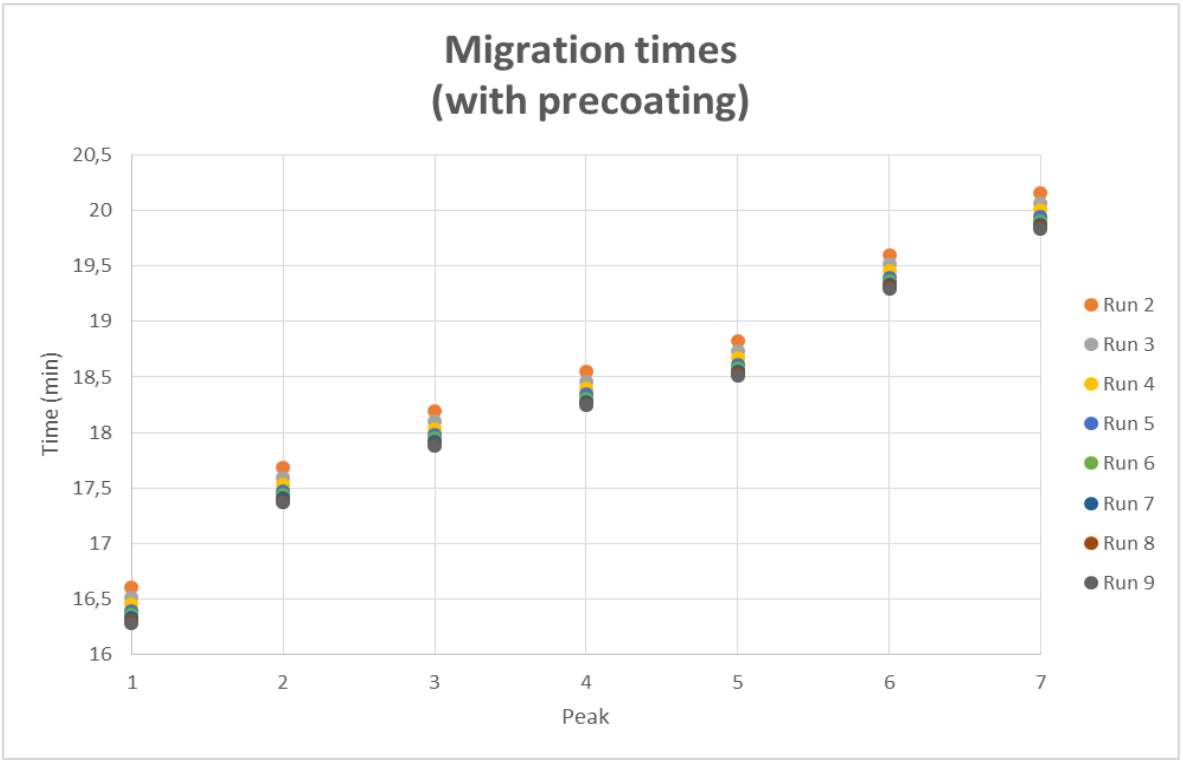

Figure A8. The migration times of the peaks from the repeated runs using a method with 1% PVP as precoating.

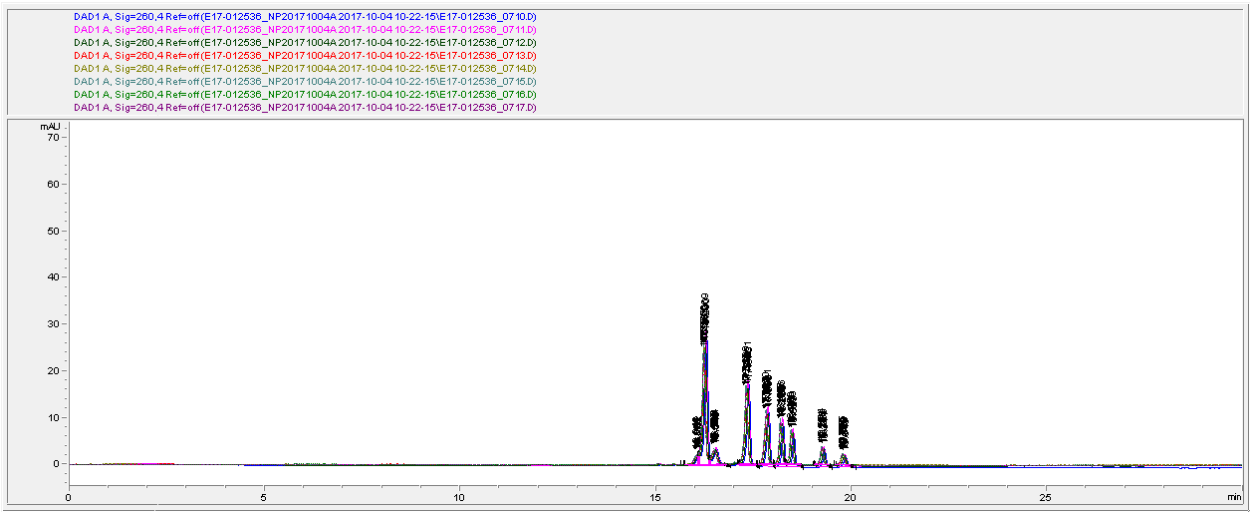

Figure A9. **Method NOT including a precoating step** (set 1). RNA ladder 0.5 mg/ml, heated 80°C for 15 min, in FACE sieving medium in ID 50 µm, effective length 40 cm capillary at 25°C. Injection parameters 80 mbar, 18 s. Applied voltage: -13 kV.

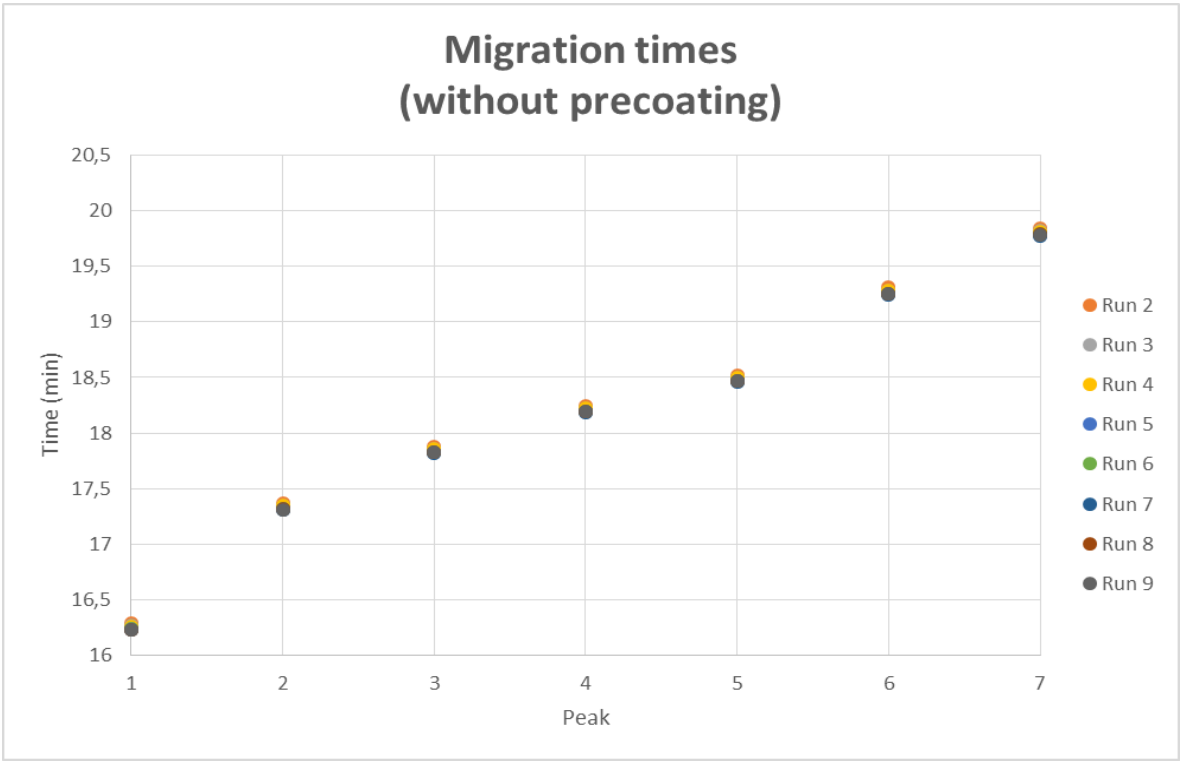

Figure A10. The migration times of the peaks from the repeated runs using a method WITHOUT a precoating step in the method (set 1).

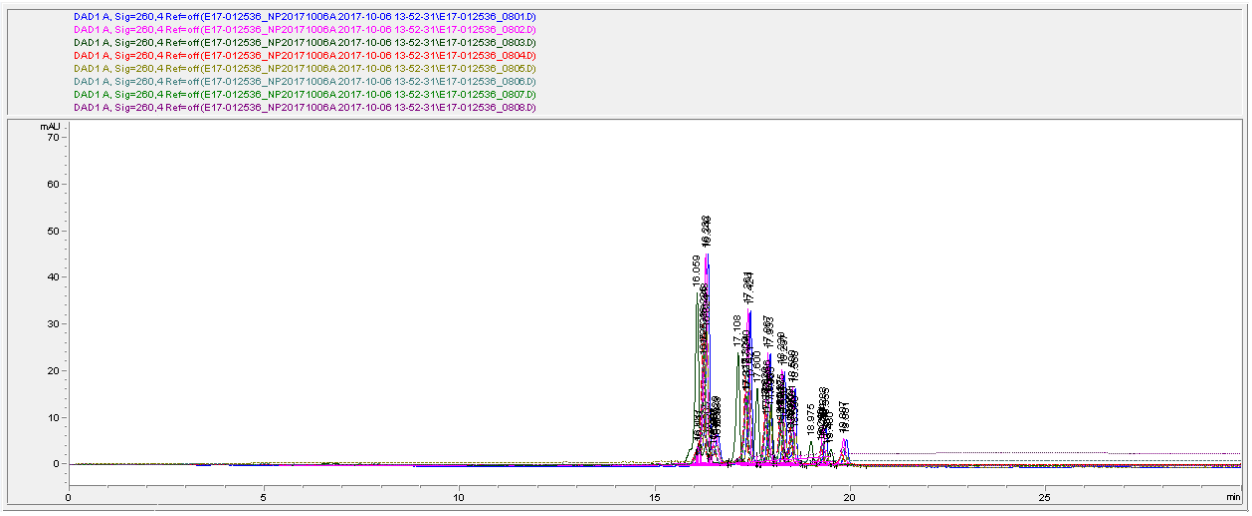

Figure A11. **Method NOT including a precoating step** (set 2). RNA ladder 0.5 mg/ml, heated 80°C for 15 min, in FACE sieving medium in ID 50 µm, effective length 40 cm capillary at 25°C. Injection parameters 80 mbar, 18 s. Applied voltage: -13 kV.

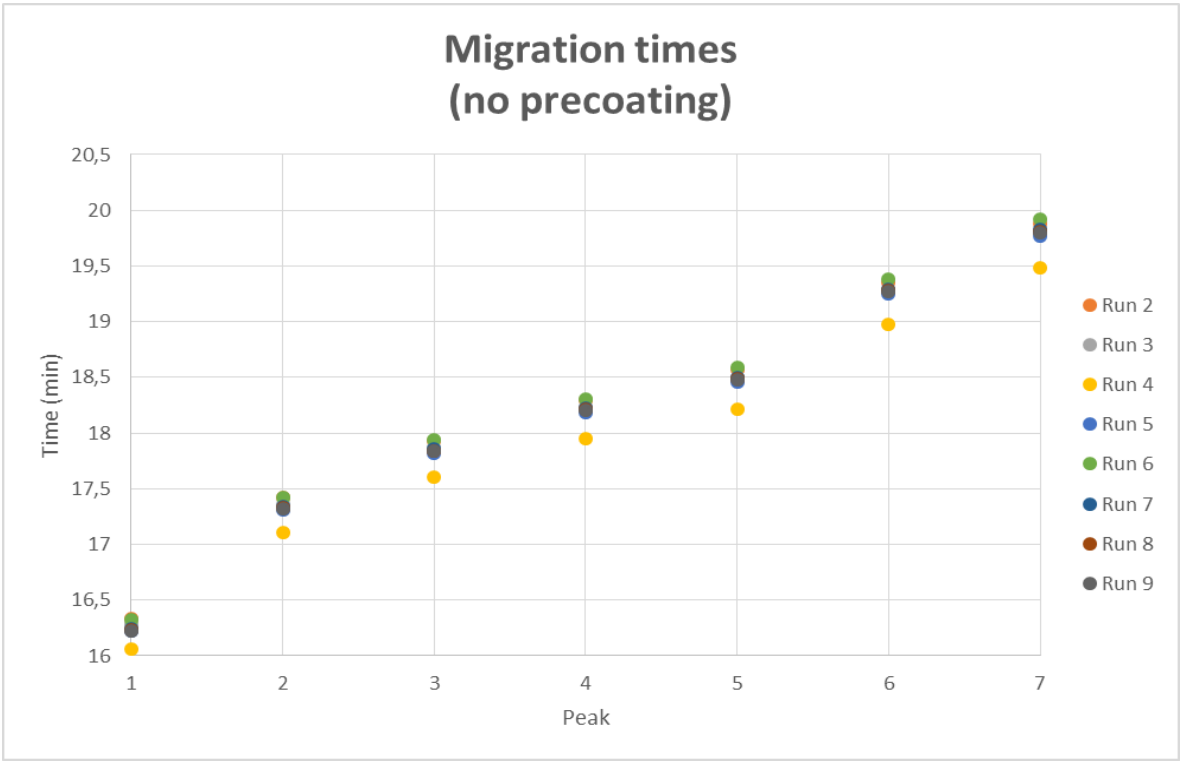

Figure A12. The migration times of the peaks from the repeated runs using a method WITHOUT a precoating step in the method (set 2).

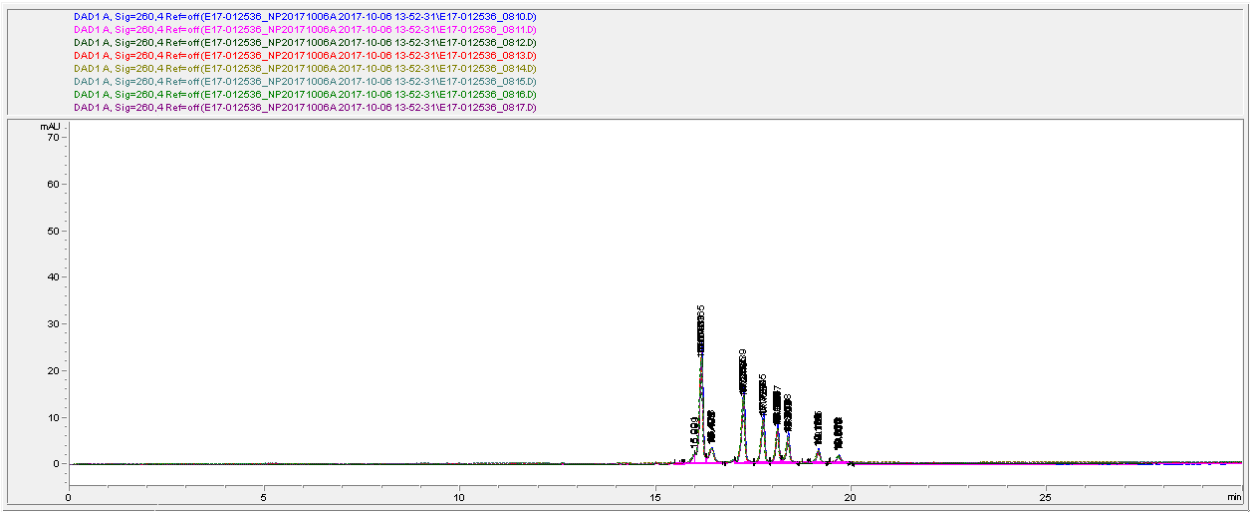

Figure A13. Method NOT including a precoating step and including a set of heavy wash steps. RNA ladder 0.5 mg/ml, heated 80°C for 15 min, in FACE sieving medium in ID 50 µm, effective length 40 cm capillary at 25°C. Injection parameters 80 mbar, 18 s. Applied voltage: -13 kV.

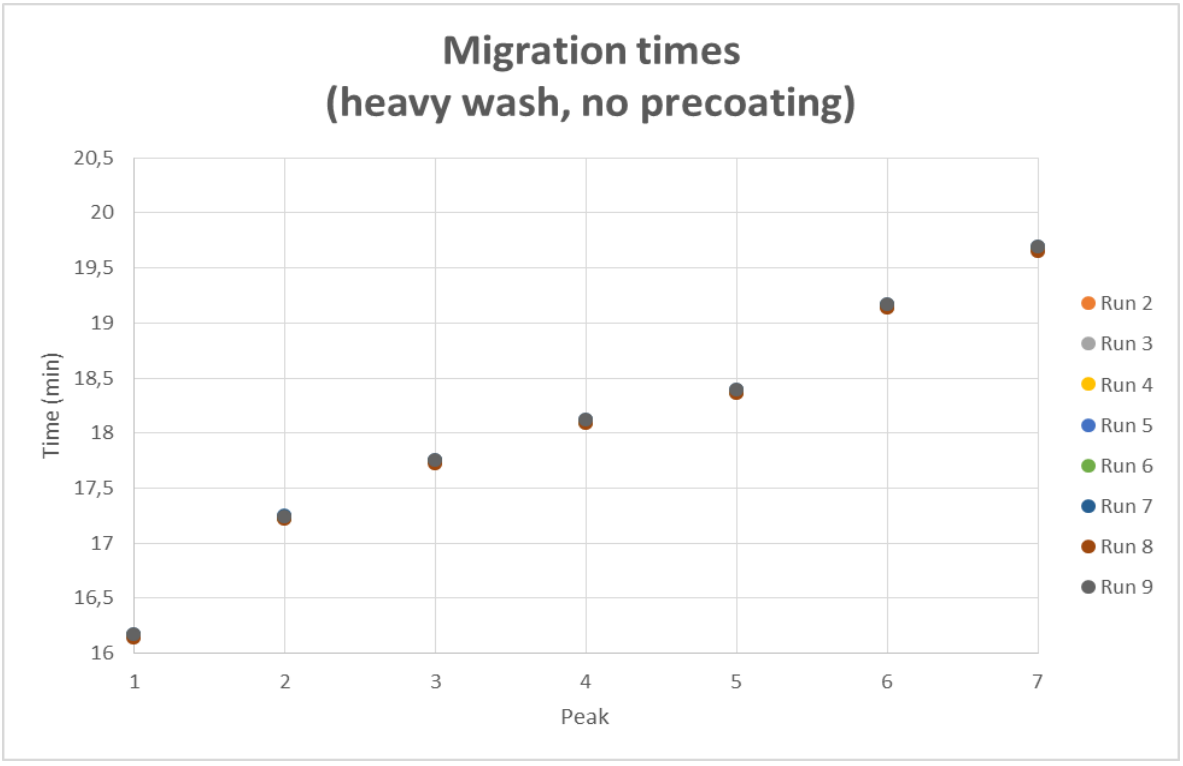

Figure A14. The migration times of the peaks from the repeated runs using a method WITHOUT a precoating step in the method but including a set of heavy wash steps pre-analysis.

Table A1. Same table and set of numbers as shown in "4.1.3 Prewash method" (*table 4*) however now excluding run 4 of both method runs as this was particularly deviating in the "No precoat"-method of this set of runs and might thereby be affecting the migration time deviation calculations in a non-representative way.

2nd set	*No precoat*							*Heavy wash, no precoat*						
Calculations	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**
Average	16,3	17,4	17,9	18,2	18,5	19,3	19,8	16,2	17,2	17,7	18,1	18,4	19,2	19,7
Standard deviation	0,044	0,044	0,044	0,044	0,045	0,046	0,048	0,007	0,009	0,009	0,010	0,010	0,012	0,012
Relative standard deviation (%)	0,269	0,254	0,247	0,240	0,241	0,241	0,244	0,045	0,050	0,052	0,054	0,055	0,061	0,062

Appendix C

Urea as sample additive

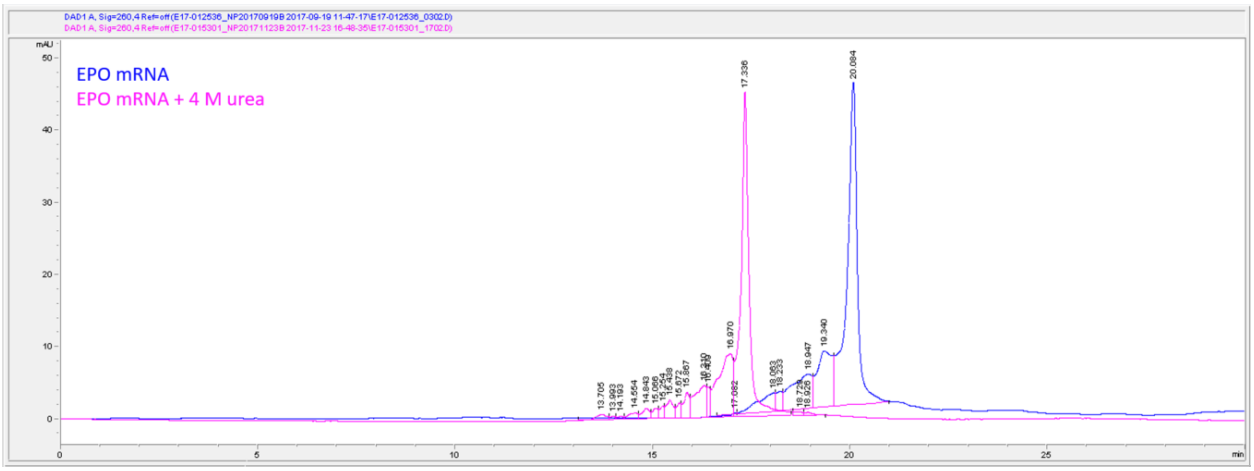

Figure A15. **EPO mRNA 0.5 mg/ml + 3.2 M urea compared to untreated EPO mRNA sample**. Both samples heated 80°C for 15 min, in separation gel 1 in ID 50 µm, effective length 40 cm capillary at 25°C. Injection parameters 80 mbar, 18 s. Applied voltage: -13 kV.

Appendix D

$MgCl_2$ as sample additive

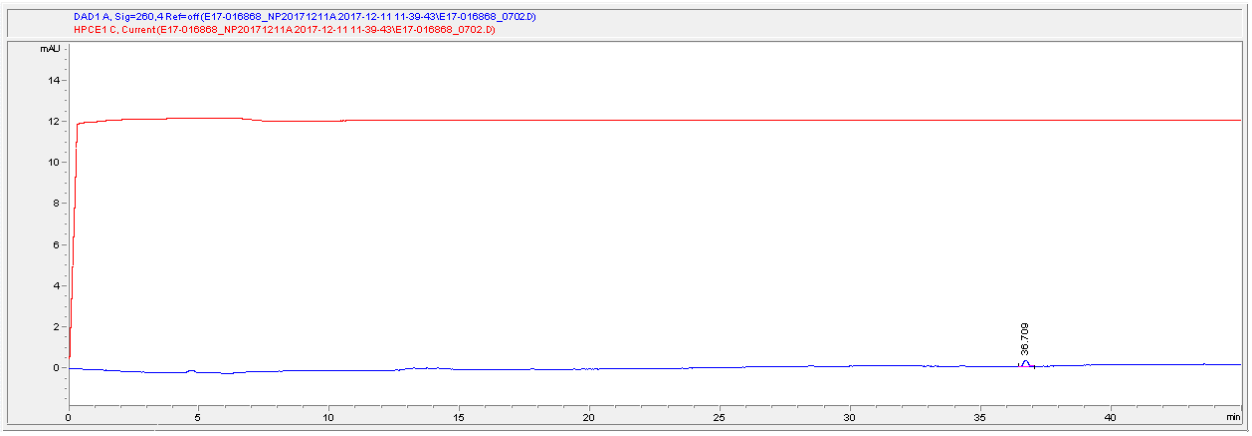

Figure A16. **EPO mRNA 0.5 mg/ml + 20 mM $MgCl_2$**, heated 80°C for 15 min, in separation gel 1 in ID 50 µm, effective length 40 cm capillary at 25°C. Injection parameters 80 mbar, 18 s. Applied voltage: -13 kV.

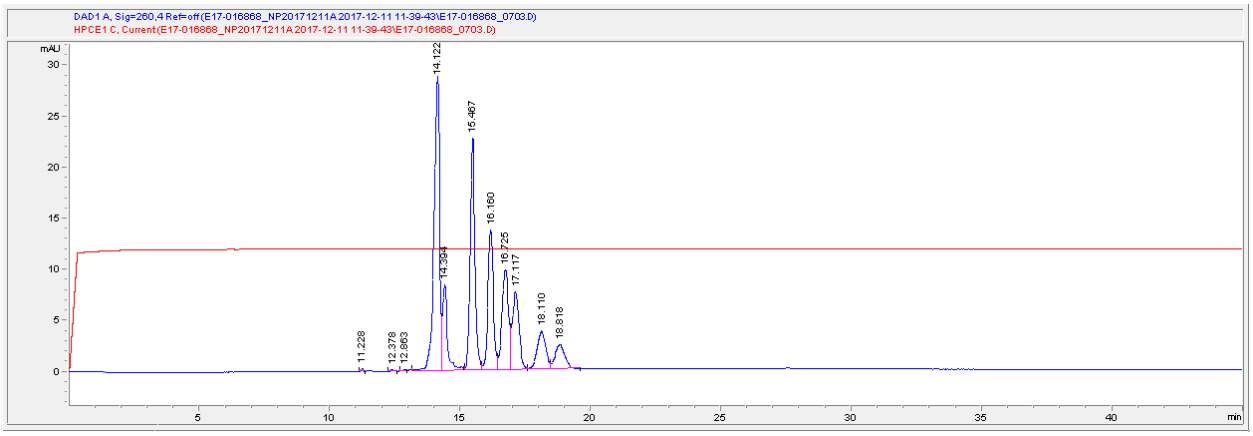

Figure A17. RNA ladder 0.5 mg/ml, heated 80°C for 15 min, in separation gel 1 in ID 50 µm, effective length 40 cm capillary at 25°C. Injection parameters 80 mbar, 18 s. Applied voltage: -13 kV.
Ordinary separation affected by the previous run containing $MgCl_2$ as sample additive.

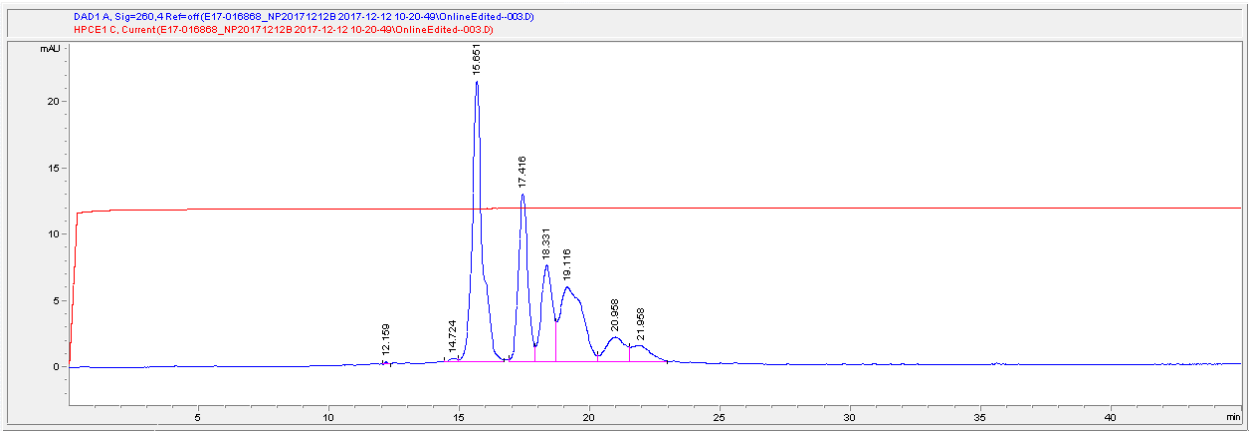

Figure A18. RNA ladder 0.5 mg/ml, heated 80°C for 15 min, in separation gel 1 in ID 50 µm, effective length 40 cm capillary at 25°C. Injection parameters 80 mbar, 18 s. Applied voltage: -13 kV. **Ordinary separation affected by the previous run containing $MgCl_2$ as sample additive even after the substitution of all vials in the system (except untreated RNA ladder sample vial). Capillary had to be replaced.**

Appendix E

HEC

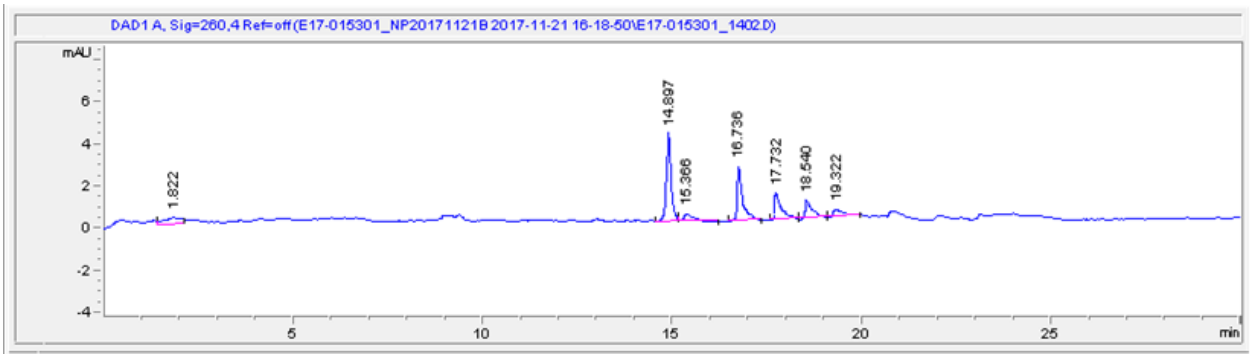

Figure A19. RNA ladder 0.5 mg/ml, heated 80°C for 15 min. **HEC 90 kDa sieving medium in permanently coated µSiL-DNA capillary** ID 75 µm, effective length 40 cm. Injection parameters 30 mbar, 9 s. Applied voltage: -13 kV.

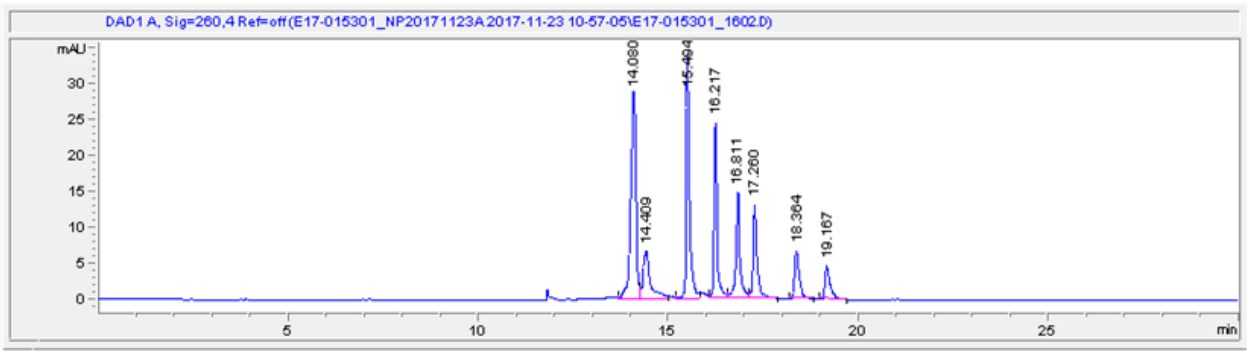

Figure A20. RNA ladder 0.5 mg/ml, heated 80°C for 15 min. **50/50 PVP/HEC 90 kDa sieving medium in bare fused silica capillary** ID 50 µm, effective length 40 cm. Injection parameters 80 mbar, 18 s. Applied voltage: -13 kV.

Appendix F

Optimal sieving medium test

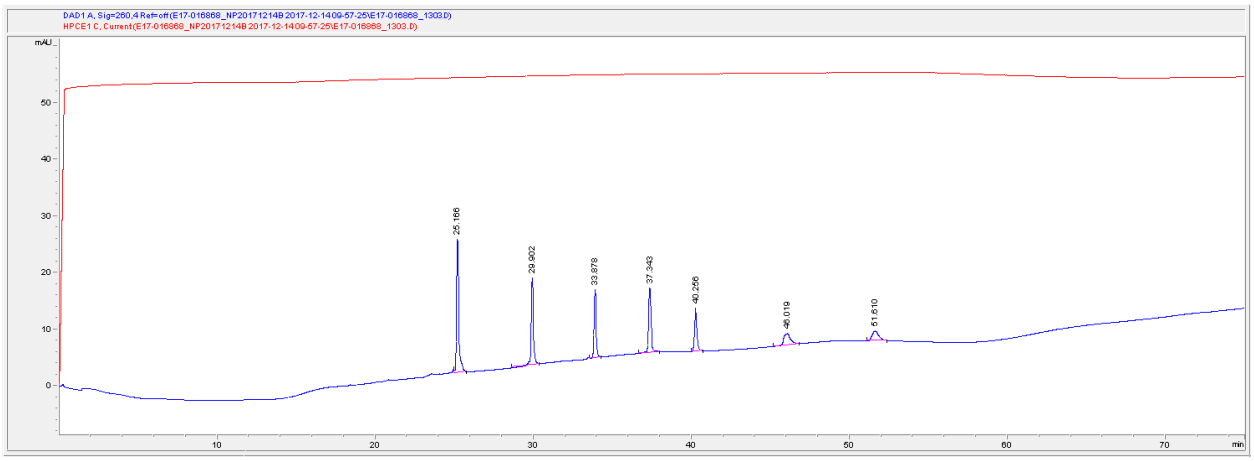

Figure A21. RNA ladder 0.5 mg/ml **in optimised sieving medium (1.32% PVP + 1.0% HEC, 10% sucrose, 15% HEPES pH 7.5, RNase free water) in ID 75 µm, effective length 104 cm capillary at 60°C**. Injection parameters 30 mbar, 22 s. Applied voltage: -30 kV.

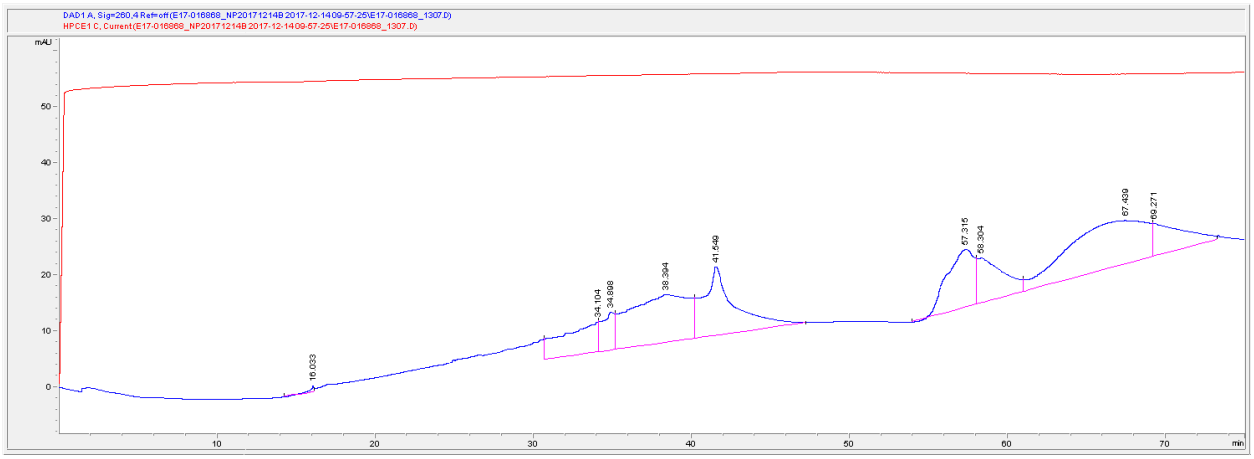

Figure A22. EPO mRNA 0.5 mg/ml **in optimised sieving medium (1.32% PVP + 1.0% HEC, 10% sucrose, 15% HEPES pH 7.5, RNase free water) in ID 75 µm, effective length 104 cm capillary at 60°C**. Injection parameters 30 mbar, 22 s. Applied voltage: -30 kV.

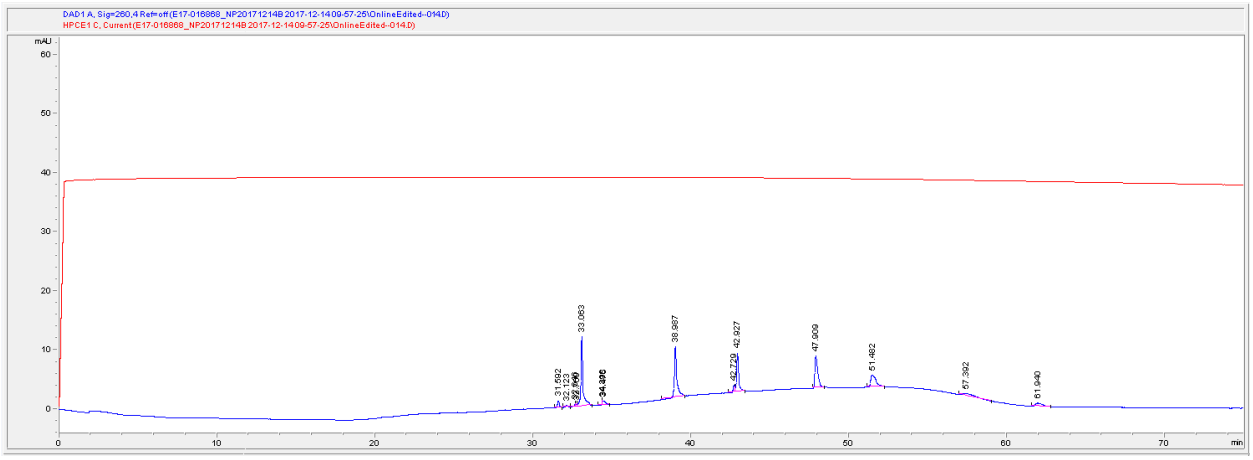

Figure A23. RNA ladder 0.5 mg/ml **in optimised sieving medium (1.32% PVP + 1.0% HEC, 10% sucrose, 15% HEPES pH 7.5, RNase free water) in ID 75 µm, effective length 104 cm capillary at 40°C**. Injection parameters 80 mbar, 30 s. Applied voltage: -30 kV.

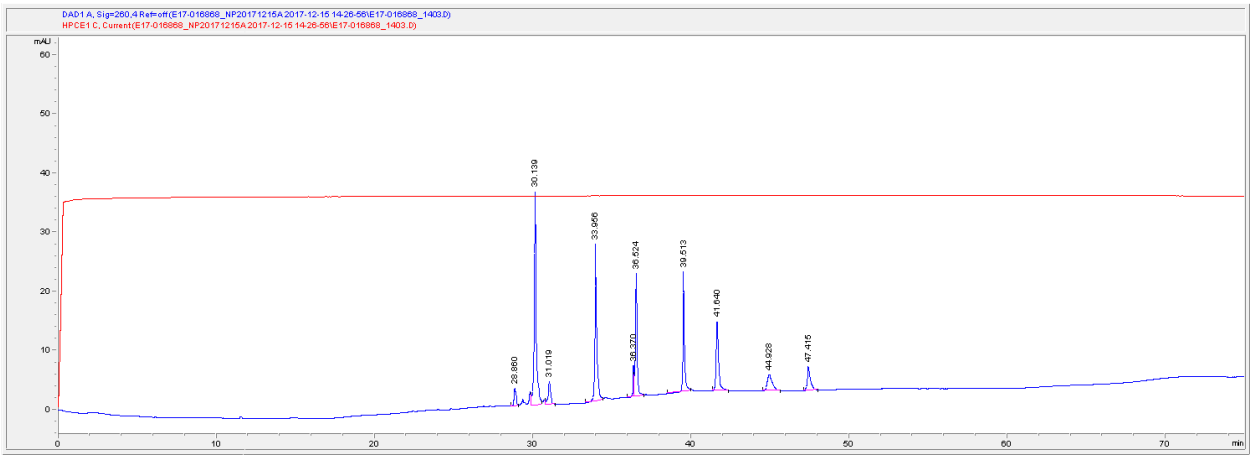

Figure A24. RNA ladder 0.5 mg/ml **in optimised sieving medium 2.0 (1.32% PVP + 0.5% HEC, 10% sucrose, 15% HEPES pH 7.5, RNase free water) in ID 75 µm, effective length 104 cm capillary at 40°C.** Injection parameters 80 mbar, 30 s. Applied voltage: -30 kV.

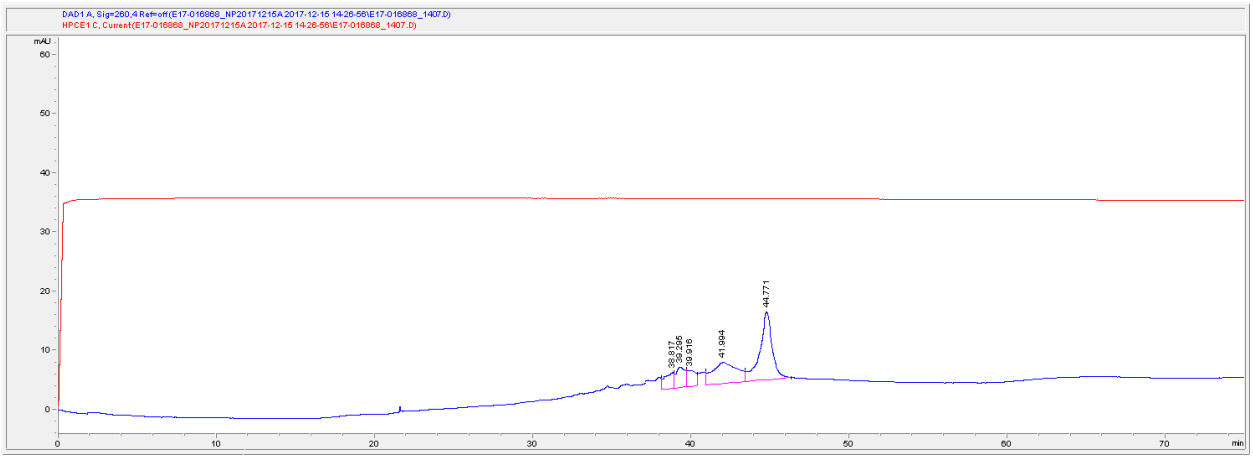

Figure A25. EPO mRNA 0.5 mg/ml in optimised **sieving medium 2.0 (1.32% PVP + 0.5% HEC, 10% sucrose, 15% HEPES pH 7.5, RNase free water) in ID 75 µm, effective length 104 cm capillary at 40°C**. Injection parameters 80 mbar, 30 s. Applied voltage: -30 kV.

Appendix G

Optimal sieving media resolution

Table A2. Resolution data of ultimate sieving medium 2.0 compared to separation gel 1 and FACE sieving medium. Capillary with ID 50 µm, effective length 40 cm at 25°C. T = migration time and the number specifies the peak (1 = 100 nt, 2 = 200nt, 3 = 300 nt, 4 = 400 nt, 5 = 500 nt, 6 = 750 nt, 7 = 1000 nt). The highest (best) resolution is marked in green.

Sieving medium	R 1-2	R 2-3	R 3-4	R 4-5	R 5-6	R 6-7
Separation gel 1	5,964	4,935	3,740	2,622	7,205	0,233
FACE	6,343	3,077	2,402	1,954	5,427	0,214
Ultimate sieving medium 2.0	8,636	7,203	4,998	3,853	11,45	0,616

Table A3. Resolution data of ultimate sieving medium 2.0 compared to other test for ultimate sieving medium. Using different capillaries at different temperatures, see table. T = migration time and the number specifies the peak (1 = 100 nt, 2 = 200nt, 3 = 300 nt, 4 = 400 nt, 5 = 500 nt, 6 = 750 nt, 7 = 1000 nt).

Sieving medium	R 1-2	R 2-3	R 3-4	R 4-5	R 5-6	R 6-7
1.32% PVP + 1.0% HEC, 10% sucrose (75 µm, 104 cm at 60°C)	17,91	15,05	12,87	9,944	10,65	2,236
1.32% PVP + 1.0% HEC, 10% sucrose (75 µm, 104 cm at 40°C)	25,46	16,71	19,27	7,986	6,662	1,819
Ultimate sieving medium 2.0* (75 µm, 104 cm at 40°C)	18,86	13,31	16,08	9,390	7,727	0,995
Ultimate sieving medium 2.0* (75 µm, 72 cm at 25°C)	11,95	8,800	5,885	4,277	11,01	1,136

*Consisting of 1.32% PVP + 0.5% HEC, 10% sucrose.