A NOVEL NITROCELLULOSE FILTER MEMBRANE BASED PARTITIONING-REVERSE TRANSCRIPTION USING REPORA-6 RNA APTAMER IN DETECTING ERYTHROPOIETIN (EPO)

PRESELA RAVINDERAN

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BY

PRESELA RAVINDERAN

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LIST OF ABBREVIATIONS

APS	Ammominum persulfate
ATP	Adenosine 5’-triphosphate
Bp	Base pair (s)
^{o}C	Degree Celsius
cDNA	Complementary DNA
CTP	Cytidine 5’-triphosphate
ddH_2O	Double-distilled water
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
eHuEPO	Endogenous human erythropoietin
et al.	and others
g	Gravitational acceleration
g	Gram
GTP	Guanosine 5’-triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hr	Hours (s)
IEF	Isoelectric Focusing
K_d	Dissociation constant
kDa	Kilodalton
M	Mol/Litre, molar
mg	Milligram
Min	Minute (s)
mL	Millilitre
mM	Millimolar
$NaOAC.3H_2O$	Sodium acetate trihydrate
ng	Nanogram

nM	Nanomolar
Nt	Nucleotide (s)
PAGE	Polyacryamide gel electrophoresis
PCR	Polymerase Chain reaction
rHuEPO	Recombinant human erythropoietin
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Room temperature
RT-PCR	Reverse transcription-PCR
s	Second (s)
SELEX	Systematic Evolution of Ligands by Exponential Enrichment
TAE	Tris-Acetic Acid-EDTA
TBE	Tris-Boric Acid-EDTA
TEMED	N,N,N',N' – Tetramethylethyleneamide
UTP	Uridine 5'-triphosphate
UV	Ultraviolet
V	Volt (s)
µg	Microgram
µL	Microlitre
µM	Micromolar
WADA	World Anti-Doping Agency

ABSTRAK

Rekombinan EPO manusia (rHuEPO), sebuah tiruan EPO endogen disalahgunakan oleh atlet dengan meluas sebagai penambah prestasi melalui satu fenomena yang dikenali sebagai pendopan. Kaedah pengesanan semasa penggunaan rHuEPO adalah melalui 'isoelectric-focusing (IEF) double-immunoblotting, Sarcosyl-PAGE (SAR PAGE) dan lateral flow test', iaitu pendekatan yang menggunakan antibodi yang dipanggil antibodi anti-EPO. Aptamers yang berasaskan asid nukleik berpotensi boleh mengurangkan kelemahan yang wujud dalam antibodi anti-EPO. Dalam kajian ini, aptamer RNA yang dihasilkan sebelum ini yang digelar sebagai REPORA-6 aptamer RNA telah digunakan untuk mengesan EPO. Objektif kajian ini adalah untuk menerima pakai REPORA-6 aptamer RNA dalam sebuah penapis filter membran berasaskan membahagi berserta dengan cerakin transkripsi terbalik dalam mengesan EPO. REPORA-6 RNA aptamer telah dicampurkan dengan Epoetin-alpha (EPO-α) sebelum penapisan melalui membran yang bersaiz 0.45 μm dengan bantuan daripada sedutan vakum. EPO-terikat aptamer RNA, yang berada pada permukaan membran telah dipulihkan semula dengan menggunakan urea, disusuli dengan etanol pemendakan. Transkripsi terbalik telah dijalankan diikuti dengan PCR sehingga muncul jaluran saiz yang betul (99 bp). Had pengesanan (LOD) ditakrifkan sebagai jumlah EPO yang menyebabkan jaluran PCR dengan intensitinya lebih tinggi daripada pengikat khusus latar belakang daripada membran yang paling rendah (0 nM EPO). Secara teknikal, EPO telah dimasukkan ke dalam serum dan air kencing untuk meniru contoh klinikal yang sebenar. LOD yang dicapai untuk EPO dan bagi EPO yang dimasukkan ke dalam serum adalah 18.38 nM. Bagi EPO yang dimasukkan ke dalam air kencing, LOD tidak dapat ditentukan kerana degradasi aptamer RNA yang mencadangkan pengubahsuain kimia untuk meningkatkan

kestabilannya. Walaupun begitu, membran berasaskan membahagi berserta dengan cerakin transkripsi terbalik berasaskan aptamer berpotensi dalam diagnostik pengesanan EPO.

ABSTRACT

Recombinant human EPO (rHuEPO), a mimetic of endogenous EPO is widely misused by athletes as a performance enhancer, a phenomenon known as doping. Current methods of detecting rHuEPO doping are via isoelectric-focusing (IEF)-double-immunoblotting, Sarcosyl-PAGE (SAR PAGE) and lateral flow test, which are antibody-based approach using anti-EPO antibody. The caveats associated with anti-EPO antibodies have spurred the interest to adopt an alternative molecular recognition element (MRE) in detecting EPO. Aptamers, as the nucleic acid-based MREs can potentially alleviate the disadvantages inherent in anti-EPO antibodies. Previously, REPORA-6 RNA aptamer was isolated against EPO with the dissociation constant of 25 nM. The objective of the study is to adopt REPORA-6 RNA aptamer in a nitrocellulose filter membrane-based partitioning coupled with reverse transcription assay in detecting EPO. REPORA-6 RNA aptamer was incubated with Epoetin-alpha (EPO-α) before filtration through the 0.45 μm nitrocellulose membrane with the aid of vacuum suction. EPO-bound RNA aptamers, retained on the surface of the membrane was recovered using urea, ensued by ethanol precipitation. Reverse transcription was carried out followed by PCR amplification till the band of the correct size appears (99 bp). Limit of detection (LOD) was defined as the lowest amount of EPO that results in the PCR band with the intensity higher than the background non-specific binders of the nitrocellulose membrane (0 nM of EPO). LOD was also approximated for the EPO spiked into human serum and urine to mimic the actual clinical samples. LOD achieved for EPO and for the EPO spiked into serum were 18.38 nM. For EPO spiked into urine, LOD could not be determined due to the degradation of the RNA aptamer, which suggests chemical modification for the enhanced stability. Despite this, nitrocellulose

filter membrane based partitioning-reverse transcription based on REPORA-6 RNA aptamer could be a potential aptamer-based assay in the diagnostic detection of EPO.

CHAPTER 1:

INTRODUCTION

1.1 Endogenous and Recombinant Erythropoietin (EPO)

Human erythropoietin (HuEPO) is a 30.4 kDa glycoprotein that stimulate the proliferation and differentiation of erythroid progenitor cells into red blood cells (Fisher, 2003). *EPO* gene which is located on chromosome 7 produces EPO, a polypeptide which contains 193 amino acids (Manneberg *et al.*, 1994) in kidney (Moore & Bellomo, 2011). *EPO* gene is activated by a stimulating factor called tissue hypoxia under stress condition in which more oxygen is needed to be supplied to the blood and artery (Figure 1.1) (Maiese *et al.*, 2004). In conditions like bleeding or haemolysis, kidney and liver cells capable of sensing the tissue hypoxia and eventually synthesise and secrete EPO into plasma (Thorling & Erslev, 1968).

The advancement of recombinant DNA technology allow researchers to isolate and incorporate the EPO-encoding genes into bacterial plasmid which was then transformed into *E.coli* host. Hence, the born of recombinant HuEPO (rHuEPO) is the first cloned hematopoietic growth factor (Jacobs *et al.*, 1985). However, the expression of rHuEPO in mammalian cells such as ovarian cells of chinese hamster (CHO) or kidney cells from hamster cub (BHK) is preferred due to the presence of disulphide bindings and glycosylation which can only be obtained in mammalian cell culture system (Choi *et al.*, 1996). Recombinant HuEPO are later on available in many forms such as EPO alpha, EPO beta and EPO omega which is primarily use for therapeutic purposes. When compared to endogenous EPO, rHuEPO has similar peptide sequence

with different sugar chains due to the lack of specific glucose transferase enzymes which function in carrying out enzymatic reactions after the formation of polysaccharides (Storring *et al.*, 1998; V. Skibeli *et al.*, 2001).

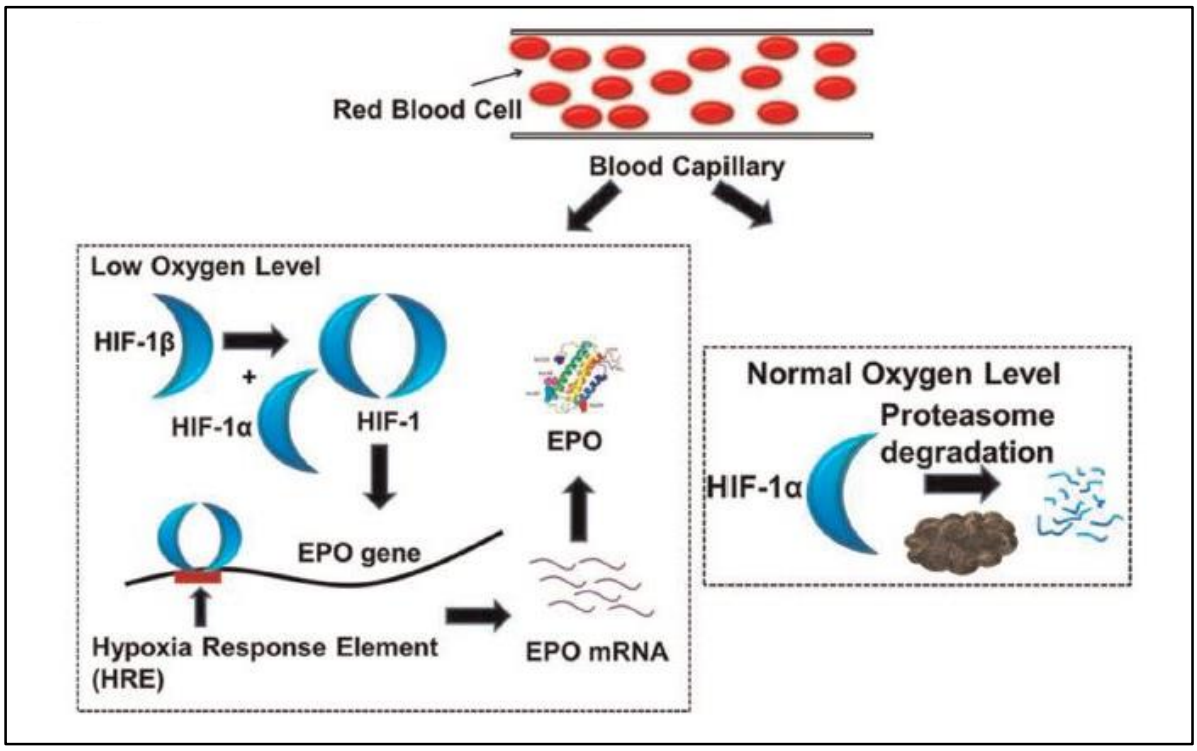

Figure Source: (Citartan *et al.*, 2015)

Figure 1.1: The interaction between tissue hypoxia and EPO**.** Under hypoxia (low oxygen level), HIF-1α forms a heterodimer with HIF-1β to generate HIF-1 which acts as the activator that binds to hypoxia response element (HRE). The binding of HIF-1 eventually increase the expression of EPO mRNA and formation of EPO. While under normal oxygen level, HIF-1α will be degraded by proteasome.

1.2 Usage of Recombinant Human EPO in Blood Doping

The capability of rHuEPO to increase haemoglobin level without any complications and difficulties such as administration without medical supervision unlike other drugs caught the attention of athletes to resort rHuEPO in increasing their stamina illegally (Bento *et al.*, 2003). This process is known as doping and many athletes are involved in doping ever since EPO is available in the market. The most publicised rHuEPO doping happened in 1998 during the "Tour de France" when rHuEPO ampules were discovered from the participating cyclists (G. Skibeli *et al.*, 1998; Zorpette, 2000). Recovery of drugs from the truck of main participating team to competitor's lodgings in this event showed the significant and adverse use of rHuEPO in sports (Lancet, 1998; Jarvis, 1999; Zorpette, 2000). The very first fatal case associated with doping occurred in 1990s when a professional Dutch cyclist died at rest due to obscure cardiac arrest while some of them died while sleeping. These cases reported are among more than 20 cases occurred during the period of 1987 to 1991 when rHuEPO was first introduced in that continent (Thein *et al.*, 1995; Gareau *et al.*, 1996).

The unexplainable fatal cases among healthy athletes are due to the uptake of rHuEPO that could place one's health under risk. This is because the increase in number of red blood cells by rHuEPO will eventually increase the blood thickness and reduce the cardiac output. In a medical condition called erythrocytic aplasia, athletes might develop anti-EPO antibodies that destroy both the endogenous EPO and rHuEPO (Casadevall *et al.*, 2002). As a result, the International Olympic Committee (IOC) banned the usage of rHuEPO since 1990 after the evidence of using rHuEPO in Calgary Winter Games and Seoul Olympic Games in 1988 was obtained. Hence, WADA

established the isoelectric-focusing double immunoblotting technique to monitor the abuse of rHuEPO among athletes (Bento *et al.*, 2003).

1.3 Direct Detection of EPO Relies on Anti-EPO Antibody

Direct detection of EPO relies on the interaction between the EPO and its molecular recognition element (MRE). The current molecular recognition element used for the detection of EPO is monoclonal anti-EPO antibody, MAb AE7A5. This antibody is used in a variety of applications such as IEF-double immunoblotting, which is the testing method accepted by WADA to detect the illegal use of rHuEPO. Moreover, another recent method approved by WADA, Sarcosyl-PAGE (SAR PAGE), which is used to monitor the detection of rHuEPO abuse among athletes, also relies on the anti-EPO antibody. Likewise, Lonnberg *et al.* (2012) have devised EPO wheat germ agglutinin (WGA) Membrane Assisted Isoform ImmunoAssay (MAIIA): a lateral flow test for rHuEPO detection that is more sensitive than IEF-double-immunoblotting, based on monoclonal anti-EPO antibody. However, non-specific interaction of this monoclonal antibody with several proteins including from human, bacterial and yeast were reported (Franke & Heid, 2006; Delanghe *et al.*, 2008). This suggests the efforts to look into an alternative MRE in order to address the specificity associated with the monoclonal anti-EPO antibody.

1.4 Aptamers-Coming of Age Molecular Recognition Element

Aptamers or also popularly known as 'chemical antibodies' are single stranded oligonucleotides that bind targets with high specificity and affinity. The word aptamer is derived from Latin word *aptus* meaning "to fit" (Ellington & Szostak, 1990; Tuerk & Gold, 1990). Aptamers bind targets via various interactions such as electrostatic, wan der Waal forces or hydrogen bonding. The simple process of in vitro selection known as SELEX is used to generate aptamers.

1.4.1 SELEX

Systemic Evolution of Ligands by Exponential Enrichment (SELEX) technology is an *in vitro* selection technique developed by Tuerk and Gold (1990) and Ellington and Szostak (1990). This technique is useful in isolating specific binding nucleic acids that bind specifically to target from pools of more than 10^{14} to 10^{15} different molecules (Jayasena, 1999).

The SELEX process begins with the preparation of the degenerate single-stranded DNA library that contains a random region flanked by a constant primer binding region at the 5' and 3' of the sequences (Figure 1.2). The library can be used directly for incubation with the target in the case of DNA aptamer generation. However for RNA aptamer generation, the ssDNA library must be PCR-amplified before subjection to *in vitro* transcription to produce RNA library. Following binding, the target-bound nucleic acid molecules are separated from the unbound molecules by partitioning method. The bound molecules are recovered and amplified by Polymerase

Chain Reaction (PCR) or Reverse Transcription-PCR (RT-PCR), for DNA aptamer or RNA aptamer, respectively. The amplicons from the RT-PCR reaction is used to generate RNA molecules for the RNA aptamer generation while ssDNA is derived from the PCR product in the case of DNA aptamer generation. These molecules are subjected to the subsequent round of SELEX and this process is repeated for 8 to 12 cycles. The nucleic acid pool is cloned, sequenced and the putative aptamer identified is verified for binding against the target protein (Kim & Gu, 2013).

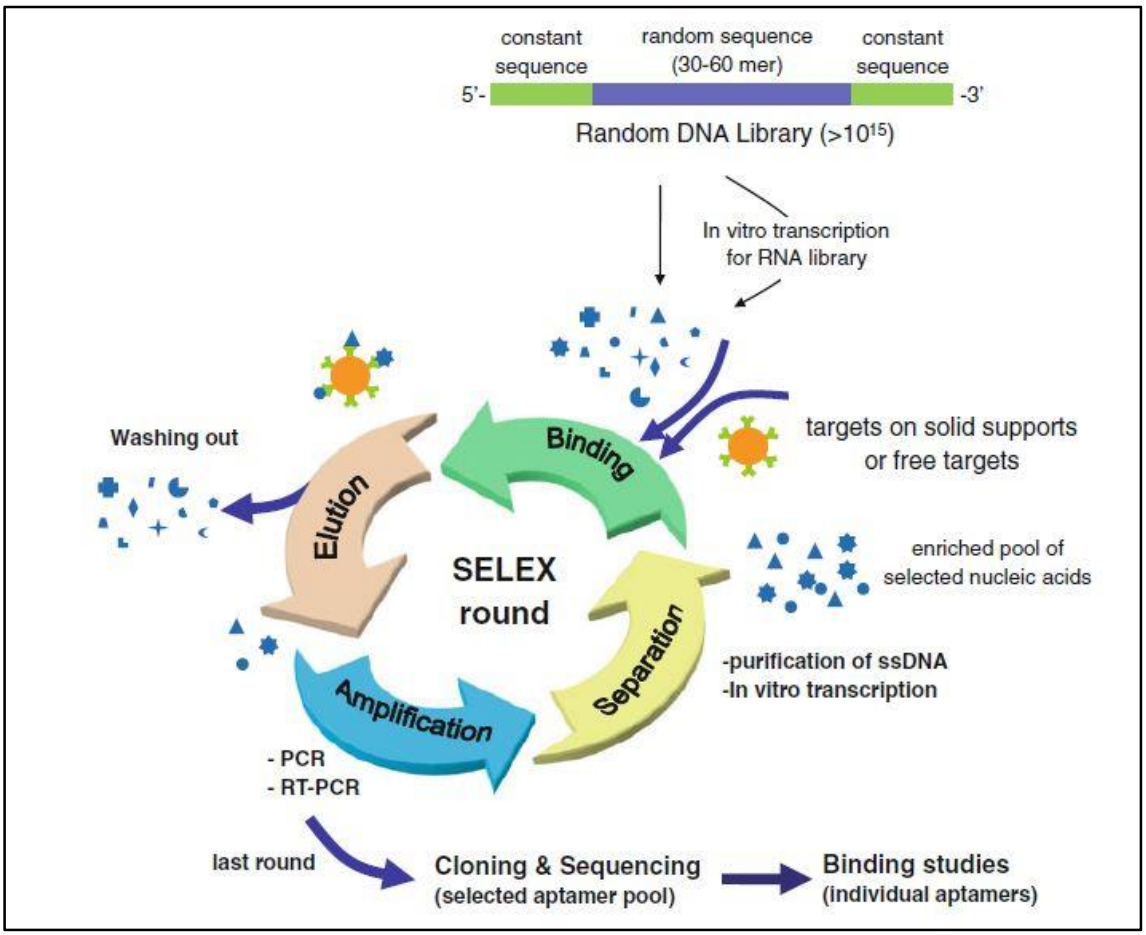

Figure source: (Kim & Gu, 2013)

Figure 1.2: SELEX technology used in the generation of aptamers. The process consists of four main steps – binding, elution, amplification and separation. Once an enriched pool of selected nucleic acids is obtained, the nucleic acids are cloned and sent for sequencing. Further binding studies will be carried out in order to determine the putative aptamer against the target.

1.4.2 Advantages of Aptamers over Antibodies

The unique properties of aptamer make it as an elegant and attractive molecular recognition element (MRE) that rival and in some cases surpass antibodies. Aptamers can detect wide variety of targets ranging from small molecules to supramolecular complexes (Jayasena, 1999; Shamah *et al.*, 2008). This includes proteins, peptides, nucleotides, amino acids, antibiotics, low-molecular organic or inorganic compounds, and even whole cells. The high specificity of aptamer enable it to differentiate between closely related target molecules. For example, aptamers able to discriminate theophylline from caffeine which differs by only a methyl group (Jenison *et al.*, 1994) and oxytetracycline from tetracycline which differs by only a hydroxyl group (Niazi *et al.*, 2008). Firstly, aptamers have a very low dissociation constant (K_d) that can go up to picomolar (1×10^{-12} M) to nanomolar (1×10^{-9} M) range, which is comparable to the binding affinity of antibody against the target antigen. Secondly, aptamers have higher thermostability than antibodies. Unlike antibodies, aptamers can resort to its original conformations at the room temperature after denaturation at much more higher temperature (Jayasena, 1999).

Besides, aptamers can be easily amplified via polymerase chain reaction (PCR) unlike antibodies, which requires a biological system for production. Hence, production of aptamer is much more cost-saving compared to the antibody. Moreover, since the production of aptamer involves *in vitro* process, there is no need for an in vivo immunization. As such, production of aptamers by using a chemical process eliminates any batch to batch variations (You *et al.*, 2003). The small size of aptamers which is usually less than 20kDa enable it to reach the previously blocked or intracellular targets

(Jayasena, 1999). Since aptamers are nucleic acid, it can be easily labelled or modified by using reporter molecules, linkers and other functional groups (Luzi *et al.*, 2005) such as dyes which acts as a simple means of detection (Jayasena, 1999). These advantages show the potentials of aptamers being used in variety of applications thus replacing antibodies.

1.5 REPORA-6 RNA Aptamer in Detecting EPO

In the previous study, RNA aptamer termed REPORA-6 specific against EPO was generated with a dissociation constant of 25±1 nM (Figure 1.3). This aptamer that can act as a 'universal probe' against all rHuEPOs/eEPO have been demonstrated to have potential in diagnostic and therapeutic applications (Citartan *et al.*, 2014).

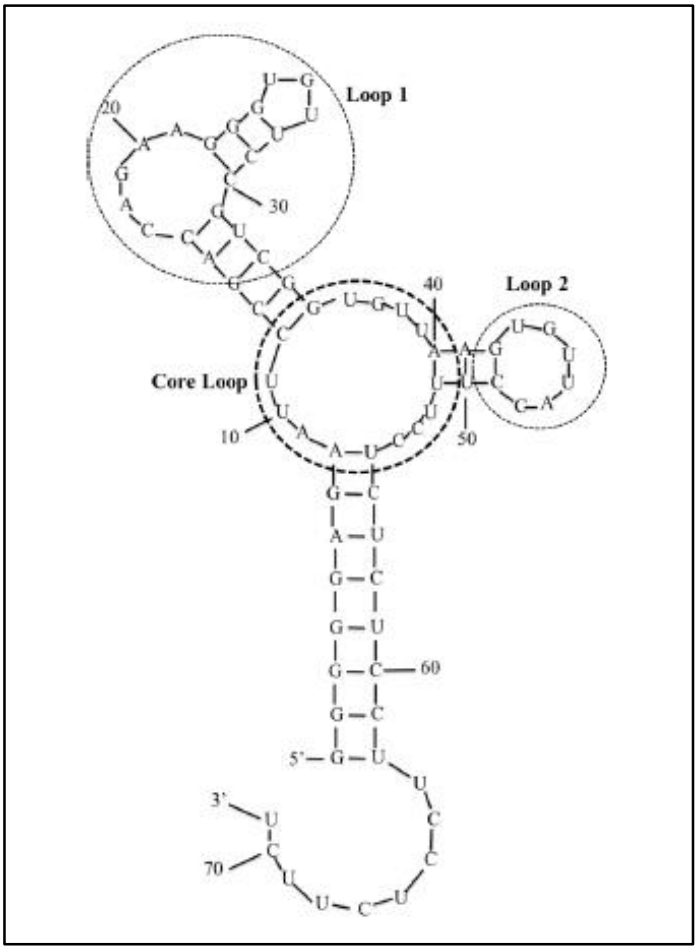

Figure source: (Citartan *et al.*, 2014)

Figure 1.3: Secondary structure of REPORA-6 RNA aptamer as predicted by mfold programme

1.6 Objectives

Though IEF-double immunoblotting is the gold standard approved by WADA, this methods suffers from several limitations. First, the usage of anti-EPO antibody is expensive. Second, cross-reactivity has been reported by Franke and Heid (2006), in which the monoclonal antibody binds non-specifically to a large number of bacterial proteins, intracellular and extracellular proteins found in urine. The caveats associated with anti-EPO antibodies suggests the adoption of a more amenable molecular recognition element (MRE) in detecting rHuEPO.

Owing to the specificity of REPORA-6 RNA aptamer against EPO, this aptamer was applied in a novel nitrocellulose filter membrane based partitioning-reverse transcription in detecting EPO. The assay developed is envisaged to have a potential for the direct detection of EPO. Thus the objectives of study are as follow:

i) To develop a novel nitrocellulose filter membrane based partitioning-reverse transcription using REPORA-6 RNA aptamer in detecting EPO

ii) To determine the detection limit of EPO spiked into human serum

iii) To determine the detection limit of EPO spiked into human urine

CHAPTER 2:

MATERIALS AND METHODS

2.1 Preparation of REPORA-6 RNA Aptamer

2.1.1 Polymerase Chain Reaction

The ssDNA oligonucleotide used in this study is 5'-GGGAGAATTCCGACCAGAAGGGTGTTCCGTCGGTGTTAAGTGTAACCTTTCCTCTCTCCTTCCTCTTCT-3' while the primers used were the forward primer-5' *AGT AAT ACG ACT CAC TAT A*GG GGG AGA ATT CCG ACC AGA AG 3' and reverse primer-5' AGA AGA GGA AGG AGA GAG GAA AGG 3'(Bio Basic Inc., Ontario Canada).

PCR amplification was carried out in a 100 µl reaction mixture containing 1X PCR buffer (10 mMTris-HCl [pH 8.8], 50 mM KCl, 0.08% [v/v] Nonidet P40) (Promega Corporation, Madison, USA), 1.5 mM $MgCl_2$(Promega Corporation, Madison, USA), 0.2 mM dNTP (Roche, Basel, Switzerland), 5 U of *Taq*DNA polymerase (Roche, Basel, Switzerland), and 0.6 µM of each primers. The amplification was carried out for 10 cycles using the PCR thermocycler (Bio-Rad Laboratories, Hercules, USA) with the following parameters; (a) denaturation at 94°C for 60 seconds (b) annealing at 55°C for 50 seconds (c) extension at 72°C for 70 seconds and (d) final elongation at 72°C for 300 seconds.

2.1.2 Agarose Gel Electrophoresis

Five microliters of the PCR product was mixed with 1 µL of 6X Blue/Orange DNA loading dye (10mM Tris-HCl [pH 7.5], 50 mM EDTA [pH 8.0], 0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll® 400) (Promega Corporation, Madison, USA) containing 0.5 µg/mL of ethidium bromide (Sigma, St. Louis, USA). Gel electrophoresis was then carried on 4% agarose gel (Promega Corporation, Madison, USA) with 1X TAE buffer (40 mMTris-acetate, 1mM EDTA) using a agarose mini gel apparatus (Bio-Rad Laboratories, Hercules, USA) at 100 V for 30 minutes followed by visualisation of the gel using Bio-Rad Gel Dol 2000 (Bio-Rad Laboratories, Hercules, USA). The size of the PCR product which is 99 bp was confirmed with 25 bp DNA ladder (Promega Corporation, Madison, USA).

2.1.3 Ethanol Precipitation

Ethanol precipitation was carried out throughout the study to concentrate both DNA and RNA. Firstly, the concentration of the salt solution was adjusted to 0.3 M by the addition of the 1/10 volume of the solution by 3 M sodium acetate (pH 5.2). Followed by the addition of 2.5 volume of absolute ethanol (Merck KGaA, Darmstadt, Germany) and incubation at -80°C for 15 min. Following centrifugation (Refrigerated Centrifuge 5810R, Eppendorf, Hamburg, Germany) at 13,000 x *g* for 15 min at 4°C, supernatant was removed and 1 mL of 70% ethanol was added as a washing step. After a brief centrifugation at 13,000 x *g* for 2 min, supernatant was removed and the pellet was air-dried in a vacuum concentrator (Eppendorf, Hamburg, Germany) and dissolved in appropriate volume of ddH2O.

2.1.4 *In Vitro* Transcription and Denaturing Urea Polyacrylamide Gel Electrophoresis

Four microliters of the ethanol precipitated PCR product was applied directly for an *in vitro* transcription reaction. *In vitro* transcription was carried out using AmpliScribe™ T7-*Flash*™ Transcription Kit (Epicentre Technologies Corp, Madison, USA). The reaction mixture assembled in 20 μL contains 1X AmpliScribe T7 reaction buffer (40 mMTris-HCl [pH 7.5], 6 mM MgCl2, 10 mMNaCl, 2 mM spermidine), 7.5 mM each of ATP, CTP, UTP, GTP, 10 mM of dithiothreitol (DTT), 20 U of AmpliScribeTM T7 Polymerase *Flash* Enzyme Solution (10 U/μL) and 20 U of RiboGuard RNase Inhibitor (40 U/μL). The reaction mixture was incubated at 37°C for overnight (Fisher Scientific International, Inc., Hampton, USA).

Following *in vitro* transcription, treatment with 10 U of RNase-Free DNase I (Fermentas, Burlington, Canada) was done at 37°C for 20 min. To stop the reaction, equal volume of 2X RNA loading dye (8 M urea, 0.05% bromophenol blue) was pipetted into the mixture. The mixture was immediately cooled on ice after heated at 95°C for 2 min in Thermo Scientific Thermo-Shaker (Thermo Scientific, Waltham, USA). The mixture was loaded onto 10%, 7 M denaturing-urea polyacrylamide gel followed by electrophoresis in 1X TBE (89 mMTris-borate [pH 8.0], 2 mM EDTA) using BioRad Mini-PROTEAN 3 Electrophoresis Cell (Bio-Rad Laboratories, Hercules, USA) at 140 V for 1 hour.

2.1.5 Purification of Transcribed RNA (Crush and Soak Method)

To purify the *in vitro* transcribed RNA, a rapid method of small RNA purification was established (Citartan et al., 2012b, 2012c). Upon electrophoresis, one of the glass plates was removed carefully and the gel was covered with a clean plastic wrap. The gel was placed upside down on a flat surface and removed slowly from the glass plate with the aid of scalpel. The gel covered with plastic wrap was placed on top of the silica coated glass plate (Merck KGaA, Darmstadt, Germany) and visualized by using hand-held u.v. light source (UVP, LLC, Upland, USA). Band of interest visualized as a single prominent band (under low-intensity u.v. shadowing [254 nm]) on the surface of the silica coated glass plate) was excised using a clean razor blade and transferred into a sterile 1.5 mL microcentrifuge tube (Eppendorf, Hamburg, Germany). The gel slices were then crushed into tiny particles by using 1 mL pipette tip. The crushed gel particles was added with 400 µL of ddH2O, heated at 50°C for 30 min, followed by the addition of 1 mL of 100% ethanol into the mixture which was then shaked vigorously. The mixture was then centrifuged at 13,000 x *g* for 1 min, followed by the collection of the supernatant into a fresh microcentrifuge tube and added with 40 µL of 3 M NaOAc (pH 5.2). After incubation at -80°C for 15 min and centrifugation (at 13,000 x *g* for 15 min at 4°C), supernatant was removed and the pellet was washed with 1 mL of 70% ethanol. Following a brief centrifugation at 13,000 x *g* for 2 min, supernatant was removed, the pellet was air-dried and dissolved in an appropriate volume of ddH2O. Spectrophotometric concentration measurement was carried out using nanodrop spectrophotometer (ImplenInc, Westlake Village, USA).

2.2 Nitrocellulose Filter Membrane Based Partitioning-Reverse Transcription

2.2.1 Nitrocellulose Filter Membrane Based Partitioning

REPORA-6 RNA aptamer was prepared as mentioned in Section 2.1. Firstly, different concentration of commercially available Epoetin-α (ProSpec-TanyTechnoGene Ltd, Israel) was prepared by diluting in 1X HEPES-KOH buffer (pH 7.4). Then 1 µg of REPORA-6 RNA aptamer dissolved in 1X HEPES-KOH buffer was denatured at 95°C for 2 mins and let to cool down at RT before adding into the EPO protein. The reaction mixture in a volume of 50 µL was incubated for 10 mins at room temperature to allow the binding to occur. The RNA protein complexed were filtered through a pre-wetted 0.45 µm nitrocellulose membrane filter (Millipore, Billerica, USA) which was placed on top of the 'pop-top' filter holder (Whatman, Maidstone, UK) with the aid of vacuum suction (Figure 2.1) (Vacuubrand, Inc., Wertheim, Germany). The membrane was washed using one mL of 1X HEPES-KOH buffer (pH 7.4) to remove the unbound molecules while the EPO-bound RNA aptamers retained on the surface of the membrane was recovered by heating in 400 µL of 7M urea (Amresco LLC, Cochran Rd, USA). This was done by adding 200 µL of 7M urea into the nitrocellulose membrane in the sterile 1.5 mL microcentrifuge tube (Eppendorf, Hamburg, Germany). The membrane was crumpled using a pipette tip prior heating at 95°C for 2 mins. This step was repeated again to recover all the bound RNA aptamers from the membrane. The solution containing the bound RNA molecules was added with 4 µL of Dr.GenTLE^{TM} precipitation carrier (Takara Bio, Japan), 40 µL of 3 M NaOAc (pH 5.2) and 1 mL of 100% ethanol. After vigorous shaking, the mixture was centrifuged immediately at 13,000 x g for 15 min at 4°C and the supernatant was discarded. The

pellet was washed with 70% ethanol and subjected to RT-PCR. For the negative control, addition of EPO protein into the 1X HEPES-KOH buffer was omitted.

2.2.2 Reverse Transcription – Polymerase Chain Reaction (RT-PCR)

The recovered RNAs from the above step was reverse-transcribed in 20 μL of a reaction mixture containing 1X AMV reverse transcriptase reaction buffer (50 mMTris-HCl [pH 8.5], 8 mM MgCl2, 30 mMKCl, 1 mM DTT) (Fermentas, Burlington, Canada), 1.6 mM dNTPs, 0.4 μM of reverse primer and 10 U of AMV reverse transcriptase (Fermentas, Burlington, Canada). The reaction mixture was heated at 95°C for 2 min following incubation at RT for 10 min before dNTPs and enzymes were added. Reverse transcription reaction was performed at 60°C for 1 hr. First-strand cDNA obtained was subjected to PCR amplification until the band of the right size (99 bp) appeared. The PCR reactions parameters were as described above (Section 2.1.1).

2.2.3 Determination of Limit of Detection (LOD)

In this assay, the limit of detection (LOD) was defined as the lowest concentration of EPO that results in the PCR product with the band intensity higher than that of 0 nM. The appearance of the PCR product at 0 nM of EPO is due to the non-specific binding of the aptamers to the nitrocellulose membrane (0 nM). The intensity of the band was measured using ImageJ programme (http://rsbweb.nih.gov/ij/).

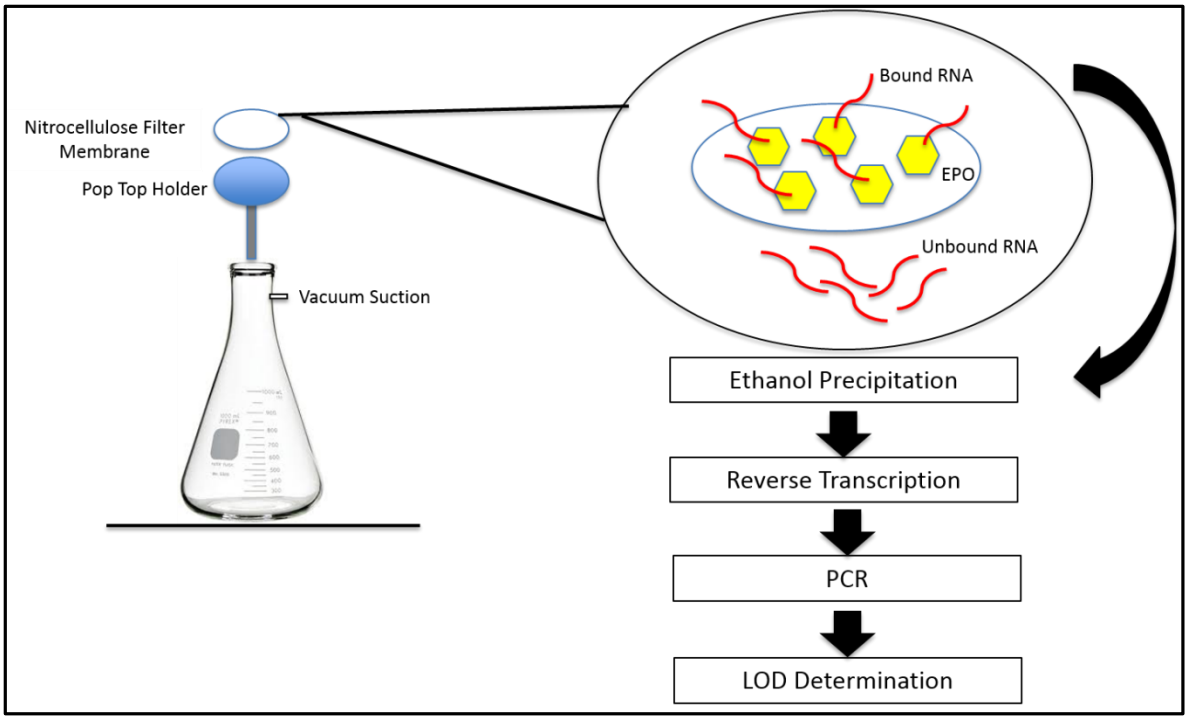

Figure 2.1: Schematic diagram on the principle of nitrocellulose filter membrane based partitioning coupled with reverse transcription in detecting EPO.

2.3 Serum Preparation and Spiking

The commercially-purchased human male serum sample (Sigma-Aldrich, St Louis, USA) was first diluted in a ratio of 1:1000 using 1X HEPES-KOH buffer (pH 7.4). The method stated in Section 2.2 was repeated. However a slight modification was done to accommodate the spiking step. Instead of adding the commercial rHuEPO-α protein directly into the HEPES-KOH buffer, the EPO protein was first mixed into diluted serum and incubated with REPORA-6 RNA aptamer before proceed to the next step (Refer to Section 2.2).

2.4 Urine Sample Preparation and spiking

The urine samples used in this project are always freshly collected in Ideal Care specimen container (Ideal Health Care, Kedah, Malaysia) and the time of the day in which the samples are collected is not fixed as it does not interfere in any way with the experiment. The urine samples obtained were centrifuged at 4000 x g (Kubota Centrifugal Machine 3740, Osaka, Japan) for 15 min at room temperature to sediment any debris present in the urine. Only the supernatant was collected in a sterile 1.5 mL microcentrifuge tube (Eppendorf, Hamburg, Germany) and used in this experiment for spiking. The collected supernatant was spiked in a similar fashion as the serum sample spiking.

2.5 Determination of REPORA-6 RNA Aptamer Stability

Urine samples obtained were centrifuged at 4000 x g (Kubota Centrifugal Machine 3740, Osaka, Japan) for 15 min at room temperature to sediment any debris present in the urine. Only the supernatant collected in a sterile 1.5 mL microcentrifuge tube was added with (1 µg) 800 nM of purified REPORA-6 RNA aptamer in a total reaction mixture of 50 µL. At 0 hour (the second EPO was added into the urine), 5 µL from the reaction mixture was pipetted into an Eppendorf tube and stored at -20 ºC to perform denaturing urea polyacrylamide gel electrophoresis. The procedure above was repeated for every 30 minutes, 1 hour, 2 hours and 24 hours at room temperature.

Equivalently, the stability of the RNA aptamer in human serum was also determined. However, the commercially-purchased human male serum sample (Sigma-Aldrich, St Louis, USA) was first diluted in a ratio of 1:1000 using 1X HEPES-KOH buffer (pH 7.4) before the addition of REPORA-6 RNA aptamer.

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CHAPTER 3:

RESULTS AND DISCUSSION

3.1 *In vitro* transcription produces intact REPORA-6 RNA aptamer

REPORA-6 RNA aptamer was derived from the amplicon of the REPORA-6 oligonucleotide ssDNA. The REPORA-6 RNA transcript was purified via small RNA purification method (Citartan *et al*., 2012b, 2012c) from the urea-denaturing PAGE. The purified RNA is stable (Figure 3.1) and is suitable for the direct detection of EPO.

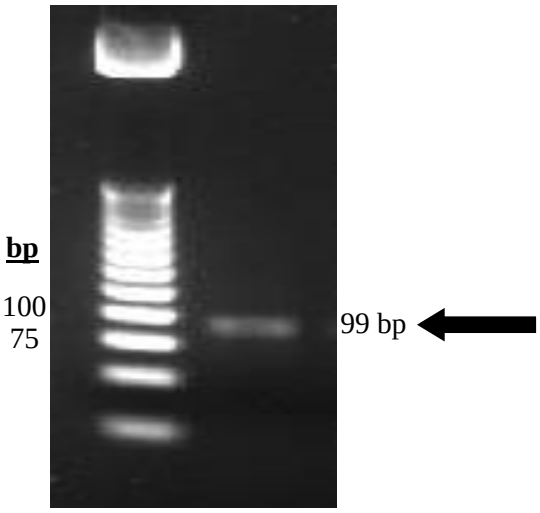

Figure 3.1: 5 µL of REPORA-6 RNA aptamer electrophoresed on 4% agarose gel.

Black arrow indicates RNA transcript.

3.2 Formation of REPORA-6 RNA aptamer-EPO complex

One µg (800 nM) of REPORA-6 RNA aptamer is incubated with EPO of different concentrations from 4.59 nM to 588.2 nM. Incubation was carried out in 1X HEPES buffer (10 mM HEPES-KOH [pH 7.4], 150 mM NaCl) at room temperature for 10 minutes. Similar binding parameters which was used previously in the SELEX for the isolation of the REPORA-6 RNA aptamer were retained for optimal aptamer-EPO complex formation. Similarly, incubation was also carried out with the EPO spiked into serum and urine samples. Incubation will result in the formation of the EPO-aptamer complex. However, the unbound REPORA-6 RNA aptamers present have to be partitioned from the EPO-bound molecules. This is important for accurate quantification of the RNA molecules bound to the EPO, as incomplete partitioning may cause false positivity. The method that is adopted for separation of the EPO-bound from the unbound molecules is the nitrocellulose filter membrane-based partitioning.

3.3 Nitrocellulose filter membrane-based partitioning to separate EPO-bound from unbound RNA molecules

Membrane filtration was developed in 1970s in the early stages of molecular biology to study the interaction of protein-nucleic acid complex formation. Initially, this method was established to examine RNA-protein interaction (Nirenberg *et al.,* 1965) before being introduced to analyse the DNA-protein interaction (Jones & Berg, 1966). In this study, following the incubation with the target EPO protein, the unbound REPORA-6 RNA aptamer is separated from the bound molecules via 0.45 µm

nitrocellulose filter membrane-based partitioning with the aid of vacuum suction. The target protein EPO will be retained on the surface of the nitrocellulose filter membrane via electrostatic and hydrophobic interactions without losing their binding capacity to the aptamers (Helwa & Hoheisel, 2010). Since the REPORA-6 RNA aptamer has a higher binding affinity towards EPO, the EPO-aptamer complex will retained on the membrane (Figure 3.2).

The retained REPORA-6 RNA aptamer on the membrane, which is bound to the EPO was recovered using urea. Recovery of the bound nucleic acids from the membrane can be carried out by heating, changing the ionic strength or pH and also by adding denaturing substances like urea, sodium dodecyl sulphate (SDS) or ethylenediaminetetraacetic acid (EDTA) (Geiger *et al.*, 1996; Bianchini *et al.*, 2001; Stoltenburg *et al.*, 2005). In this study, urea is used as the chaotropic agent as it is simple and inexpensive. Heating at 95°C for five minutes causes the urea to impart its denaturation property on the EPO protein, thus releasing the RNA molecules. The recovered RNA molecules were then ethanol precipitated and subjected to the amplification step.