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Glycerol Reduces Cross Hybridization on Nitrocellulose Membrane

Gliserol Mengurangi Hibridisasi Silang pada Membran Nitrosclulosa

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Abstrak

Lateral flow assay (LFD) berdasarkan *nucleic acid lateral flow* (NALF) adalah metode yang dikembangkan akhir-akhir ini. Metode ini sesuai dengan tuntutan *point of care testing* (POCT) yaitu mudah dan cepat dalam prosedur kerja, selain itu menggunakan peralatan yang sedikit dan dapat dilakukan oleh petugas yang tidak terlatih khusus. NALF berdasarkan hibrididasi asam nukleat lebih ekonomis daripada uji berdasarkan immunokromatografi yang menggunakan pengenalan antibodi-antigen.. Hibridisasi silang menjadi masalah utama terutama digunakan untuk membedakan 2 organisme dengan kandungan GC tinggi dan homologi yang yang tinggi karena adanya kesamaan genom yang tinggi. Beberapa teknik telah digunakan untuk memberikan kondisi *stringency* tinggi yang dapat menghindari hibridisasi silang. Teknik ini memerlukan tambahan prosedur jika digunakan. Studi ini menemukan bahwa senyawa gliserol dapat digunakan dalam dapar untuk mengurangi kejadian hibridisasi silang pada membran nitroselulose. Studi menggunakan 2 jenis larutan adasar dapar yaitu PBS dan SCC *stringency* tinggi dan konsentrasi tinggi ssDNA sebagai sampel. Tanpa gliserol memberikan hibridisasi silang pada *test line* sedangkan menggunakan gliserol dapat mengurangi bahkan menghilangkan hibridisasi silang pada penggunaan dapar dengan larutan dasar PBS. Selain itu penggunaan gliserol dapat meningkatkan sinyal hibridisasi pada dapar adengan larutan dasar SSC secara nyata ($p>0.05$)

Kata kunci: hibridisasi silang; membran nitroselulose**;** *nucleic acid lateral flow*

Abstract

Lateral flow assay (LFD) based nucleic acid lateral flow (NALF) method has been developed recently. The method met point of care testing (POCT) as a simple and rapid procedures, less equipment, and it can be performanced by less skilled technician. NALF based on nucleic acid hybridization is known more economical than immunochromatography assay which use antibody-antigen recognition. Cross-hybridization has issued while used to differentiate organism with high GC content and high homology as high similarity genome. Some techniques has applied some methods to give high stringency condition avoid cross hybridization reaction but need more procedure to apply. We found glycerol applied to buffer assay could reduce cross hybridization on nitrocellulose membrane. The study used 2 kinds of high stringency buffer there are PBS and SSC bases, , and high concentration of ssDNA amplicon as sample. Without glycerol ingredient gave cross hybridization signal on test line. But used glycerol could reduce those even omitted with PBS based buffer assay. Beside those, glycerol could significantly increased hybridization signal in SSC based buffer assay ($p<0.05$).

Key words: buffer; cross-hybridization; nucleic acid lateral flow; nitrocellulose membrane;

Introduction

Nucleic acid lateral flow has widely used for confirming result of nucleic acid amplification (amplicon). This method relies on hybridization of two complementary nucleic acid sequence, target amplicon and capture probe. The capture probe as recognition sequence immobilized on nitrocellose membrane and the amplicon as target which bind reporter flow toward immobilized capture probe. Positive result is showed by the signal of reporter, usually color, so it can be detected visually by eye. Due to simplification, rapidness, and uninstrumentation, so that it meet POCT requirement as well as sensitivity and specificity.

Hybridization is a process for binding of two complementary nucleic acid sequence thorough interactions between distinct nucleobases. This method has been used as a tool in recognition technique. It is applied widely while combining with other process such as nanoparticles technology as a simple and rapid procedure (Kusumawati *et al*., 2015; Lyberopoulou *et al*., 2016).. Antibody-antigen recognition method as a similar technique was developed, but it is so complicated procedures and high difficulty as well as high cost. So, the hybridization method is more economic **(**Jauset-Rubio et al., 2016).

Cross hybridization occurs when non specific target DNA bind to capture probe, and it it give false positive signal. It occur if the capture probe has high similarity or homology to non target (>70%), long of 100% homology continue sequence, and high GC content (>55%) (Kucho *et. al*., 2004). Base on this fact, the sequence of capture probe must be designed to avoid them.On the other hand, good capture probe is not qguaranty for the excess of cross hybridization as a result of environment condition also has important role for the success of hybridization. Buffer is known known give suitable environment for required hybridization that is categorized as low, medium, and high stringency as well as temperature of hybridization (Sambrook and Russel, 2001).

It is more difficult to differentiate two or more organism which has high similarity genome related to isolate or serotype and high GC content. Specific sequences have to chosen with compromising of homology to non target, long of 100% homology continue sequence, and high GC content to avoid cross hybridization (Kucho *et. al*., 2004). Beside those, it needs a environment to give high stringency using low monovalent cation buffer and higher temperature but it will decrease rate of hybridization (Farrell-Jr, 2010). Low monovalent cation buffer is not always works to omit cross hybridization especially at high concentration of DNA target and low temperature (Sambrook and Russel, 2001). Some chemical reagents such as formamide and DMSO are usually used to increase the specificity that is known by decreasing melting temperature (Tm) mechanism. Formamide olso still uses in buffer hybridization until now (Wang *et al*., 2014).

Hybridization influenced by macroscopic and microscopic viscosity environment that changed of time assay. Microscopic viscosity decreaseing renaturation rate of duplex DNA (Anderson and Young, 1985). On the other hand, mmicroscopic viscosity will increase the renaturation rate by increasing micro concentration of DNA sample as a result of exclusion out from water solution (Chang *et al*., 1974). Glyserol is a organic compound with low molecular weight which change viscosity of buffer assay. The increasing of viscosity lead to the decreasing of velocity which gave adequate time to the two complementary nucleic acid sequence to hybridize.

Materials and Method

Capture probe and ssDNA target

The study used 4 sequences of oligonucleotide (27-28 base lenght) that is showed in Tabel. 1). Those sequences derived from each serotype (1-4) of dengue virus. Those sequences originated from the same region of dengue genome with high similarity (55,5-100%) to ssDNA sample. Thee regions have high GC content (51-71%) and length of continue 100% homology to non target (9-13 nt).

Samples that used in the study was ssDNA with 97 nt in lenght and labeled with biotin at 5' end. Sample ssDNA was anti sense orientation of serotype 1 dengue virus genome that 100% complementary to the capture probe D1 and 55.5 to 64% to the others (D2-D4). All capture probes and ssDNA were supplied by Integrated DNA Technology (USA).

Table 1. Oligonucleotide sequence used in this study

Sequence	Sequence 5'− 3'
D1	**CACCAGGG**GAAGCTGTA**CCCTGGTGGT**
D2	**GGTGAGAT**GAAGCTGTA**GTCTCACTGG**
D3	**GCACTGAGG**GAAGCTGTA**CCTCCTTGCA**
D4	**AGCCAGGAG**GAAGCTGTA**CTTCTGGTGG**
ssDNA	Biotin-GGTCTCCTCTAACCTCTAGTCCTT*ACCACCAGGGTACAGCTTCCCCTGGTG*TTGGGCCCCGCTGCT GCGTTATGTTTCGGGAGGGGTCTCCTC TAACC

Note: Specific bases for serotype printed in bold; sequence complementary to D1 in italic

Tabel 2. Oligonucleotide characteristics

Capture Probe	Length (nt)	GC content (%)	Tm (^{O}C)	Homology to ssDNA (%)	Length of 100% continue sequence complementary to ssDNA
D1	28	61.7	48.5	100.0	28
D2	27	51.8	43.9	55.5	9
D3	28	57.1	47.1	64.3	13
D4	28	57.1	47.1	57.1	11

Notes: Tm: melting temperature calculated using Sambrook and Russel (2001) with Na+ 0,068 mM (PBS 0,5x), 1 base mismatch 1 base and 15% formamide 15%

DNA dipstick assembly

One hundred micro liter of each capture probes were immobilized into test pad (nitrocellulose membrane, Merck-Millipore, India) at speed of 0,06 µL/mm dispense rate using Isoflow dispenser (Imagine Technology, USA) in order to gave 0.6 mm wide on nitrocellulose membrane. Gold nano particles conjugated streptavidin (AuPNs-SA) (15 nm, OD 20) was spread on nylon membrane at 2x0,25 µL/mm dispense rate. Then, they were dried in an hour in dehumidifier. Stripped capture probes were immobilized on membrane by cross linking using UV lamp (30 W, 256 nm) (G30T8 Phillip, USA) for 120 seconds. Nylon membrane, nitrocellulose membrane and absorbent paper were assembled on backing plastic sheet. Then, it cut into 4 mm wide (Kinematic Automation, USA) and stored in closed packing at dry condition.

Hybridization assay

Assay was conducted with both sample ssDNA (100, 50, 25, 12.5 pmol) and 100 µL buffer assay at room temperature (25-30^{o}C). A quantity of sample was dropped into nylon membrane followed by 100 µL buffer assay. Buffer components were BSA (Sigma, USA), Tween20 (Sigma, USA), sucrose (Nacalai Tesque, Japan), glycerol (Merck, German), SDS (Sigma, USA), TritonX100 (Sigma, USA), PBS (Nacalai Tesque, Japan). Buffer PBS 0.5x ingredients were 0,5% BSA, 0,125% Tween-20, 5% sucrose, 15% formamide in 0.5x PBS solution. Buffer 0,5x SSC solution were SDS 0,1%, tritonX-100 1,4%, formamide 15% in 0.5x SSC solution. Both sample and buffer flowed laterally with AuPNs-SA to upper section of dipstick DNA, test pad and absorbent pad. Hybridization of ssDNA target with capture probe occurred and color signal on test line of nitrocellulose membrane rose in minutes. It waited until 90 minutes until the reaction finished. All assay tests waswere conducted triple. Color signal was scanned by commercial scanner (Hp Deskjet 2600) and the quantification of color intensity was conducted using ImageJ software (NIH, USA). The difference effect of color signal with different buffer solution was analyzed using Anova one way with SPSS 16 software. Cross hybridization occurrence on each test line was confirmed visually by naked-eye.

Results and Discussions

Result of study showed cross hybridization was occurred on D3 and D4 test line using PBS 0,5X and D4 using SSC 0.5X buffer assay.

This phenomenon was predicted as a result of some mismatches between two complementary nucleotide sequences. A small part of nucleobase cannot generate hydrogen bonding to a target,. but, the others the adjacent nucleobases still bind to form base-pairing to complementary bases as target. Distribution of mismatch position in probe or target gives effect on hybridization. Possibility of cross hybridization is less if bases mismatch was distributed along or in central sequence (Letowski *et al*. 2004). Mismatch bases at position 5' and/or 3' end of sequence will give more possibility to occure of cross hybridization. Capture probes (D1, D2, D3, and D4) had specific bases to serotype or mismatch bases to non-target serotype where were distributed at 5' and/or 3' end of sequence (Letowski *et al*. 2004) . On the other side, each probe sequences shared 9-13 bases in central sequence that contain 100% homology continue as conserved region. Sequence with less than 14 bases length did not showed cross hybridization while having less 51% GC contain and 75% homology (Kane *et al*., 2000). Another study claimed 18-23 bases did not show cross hybridization which sequence that had less than 51% homology and 55% GC contain (Kucho *et al*., 2004).

Low stringency condition led to mismatch base pairing. If the hybridization temperature is so belower than melting temperature, it will caused nucleobases making the formation of unspesific base-pairing. It is known easier to form nucleobase binding at low temperature spontaniously,. although there some uncomplimentary bases are distributed along sequence, and two of less complementary sequences can be hybridized untighly. In contrary, at higher temperature, the ability of nucleobase to bind will decrease, so that only nucleobase with higher binding energy can be forming base pairing. The binding strength can be showed by Gibss free energy (ΔG) which calculated from the number of base-pairing along sequence (Gao *et al*. 2006). If the value of ΔG is minus, it showed the energy is spontaneously to formt duplex at certain temperature. In oppositely, while Gibss free energy (ΔG) has positive value, that means is a energy to release the formation of duplex at certain temperature. This energy also depends on temperature. In the higher temperature, is known will decrease of energy. Sometimes, at

Table. 3. Color signal visual inspection of buffer solution test using ssDNA DENV1 in triple repeating

Buffer	Sample Consentration (pmol)	Color Signal											
		Test Line D1			*Test Line* D2			*Test Line* D3			*Test Line* D4		
		Repeating			Repeating			Repeating			Repeating		
		1	2	3	1	2	3	1	2	3	1	2	3
PBS 0.5X	100	+	+	+	–	–	–	+	+/–	+	+	+/–	+
	50	+	+	+	–	–	–	–	–	–	+/–	–	+
	25	+	+	+	–	–	–	–	–	–	+	+/–	+
	12.5	+	+	+	–	–	–	–	–	–	+	+/–	+/–
	6.25	+/–	–	+/–	–	–	–	–	–	–	–	–	–
PBS 0.5X + 15% Glyserol	100	+	+	+	–	–	–	–	–	–	–	–	–
	50	+	+	+	–	–	–	–	–	–	–	–	–
	25	+	+	+	–	–	–	–	–	–	–	–	–
	12.5	+	+	+	–	–	–	–	–	–	–	–	–
	6.25	+/–	+	+	–	–	–	–	–	–	–	–	–
SSC 0.5X	100	+	+	+	–	–	–	–	–	–	–	+/–	–
	50	+	+	+	–	–	–	–	–	–	–	+/–	–
	25	+	+	+	–	–	–	–	–	–	+/–	–	–
	12.5	+	+	+	–	–	–	–	–	–	+/–	–	–
	6.25	+	+/–	+/–	–	–	–	–	–	–	–	–	–
SSC 0.5X + 15% Glyserol	100	+	+	+	–	–	–	–	–	–	+/–	–	–
	50	+	+	+	–	–	–	–	–	–	+/–	–	–
	25	+	+	+	–	–	–	–	–	–	–	–	–
	12.5	+	+	+	–	–	–	–	–	–	–	–	–
	6.25	+/–	+/–	+/–	–	–	–	–	–	–	–	–	–

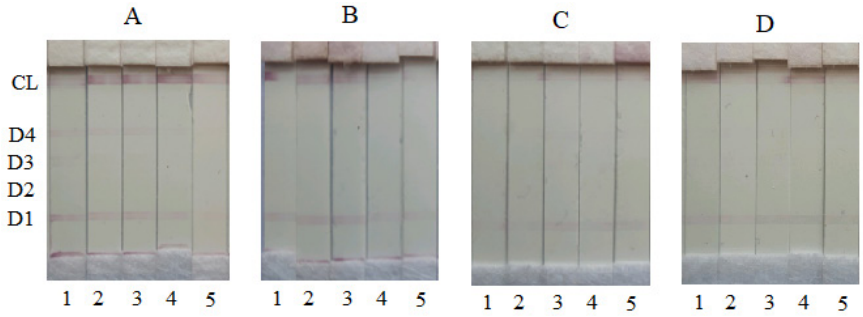

Figure 1. Color signal on NALF. A: PBS buffer; B: PBS+Glyserol buffer; C: SSC buffer; C:SSC+Glyserol buffer; 1, 2, 3, 4, 5 were 100, 50, 25, 12.5, 6.25 pmol of DENV1ssDNA.

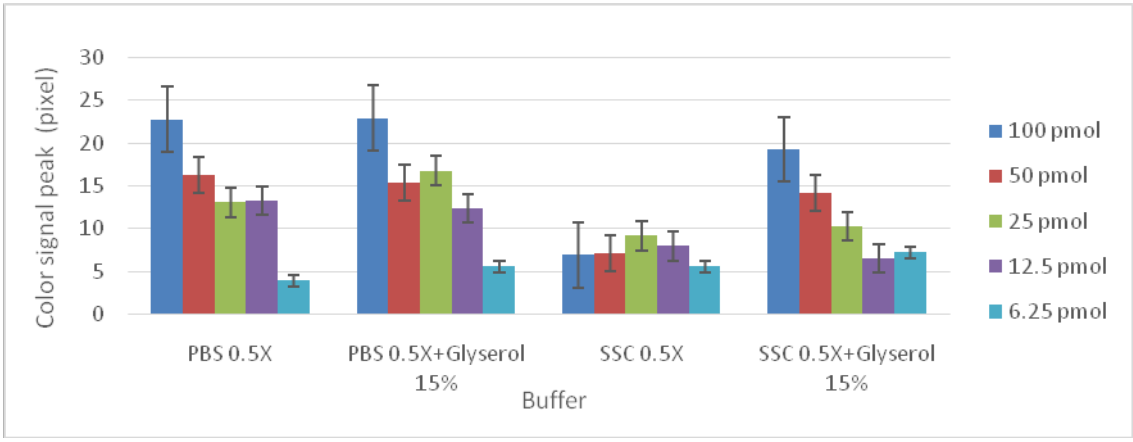

Figure 2. Color signal intensity in variation type of buffer solution using ssDNA DENV1 sample

higher temperature, the binding of complementary sequence still exist as a result of its high binding energy so that it give more matched base-pairing (Weckx *et al*., 2007). NALF method that is preferred to use at room temperature hybridization in order to pullfil of POCT requiring, so that it was needed the strategy to compromise all condition that effected NALF performance (specificity and sensitivity). Several strategy such as probe design, buffer, and temperature. In this study, probe and temperature was fixed caused of the nature of genome and its goal of application. These facts drove buffer as a strategy that was predicted becoming factor influencing performance.

The result show, very low monovalent cation (0.068 mM Na^+) and high concentration of formamide (15%) in PBS and SSC based buffer assay was still giving cross hybridization on D3 and D4 test line. Those reagent composition keep highly stringency as a result of decreasing Tm which is showed in Tm calculation (Sambrook and Russel, 2001). Hybridization reaction at room temperature gave cross hybridization. But, addition of 15% glycerol to PBS and SSC based buffer assay showed reducing of cross hybridization on D3 and D4. Cross hybridization did not occur at the treatment with PBS + glycerol all for all sample concentration. The use of SSC + glycerol, showed cross hybridization did not occur at a lower sample concentration (<50 pmol) (Table. 3). Glyserol could increase color signal significantly ($p<0.05$) at SSC based buffer assay. The result was in line with previous study, Reinhartz *et al*. (1993) and Dave, N. and Liu (2010), showed glycerol increased efficiency hybridization by controlling velocity of flowing buffer assay and volume of target sample moved to immobilized probe (test line). Addition of glycerol would increase viscosity and reduced the flowing speed of buffer assay and also the volume migration of target sample. These phenomena might reduce the formation of cross hybridization. The result can be seen on the the consistency of color signal for concentration level at SSC+glycerol buffer (Fig.2) compared with SSC bases buffer without glycerol.. This phenomena might be as the other effect of glycerol addition to buffer hybridization.

Conclusions

Glyserol in hybridization assay buffer could reduce cross hybridization on membrane nitrocellulose. This reagent could applied to avoid cross hybridization issue that led to false positive interpretation especially for hybridization method to NALF assay that is usually appllied at room temperature..

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