Glycerol Reduces Cross Hybridization on Nitrocellulose Membrane

Gliserol Mengurangi Hibridisasi Silang pada Membran Nitrocellulose

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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 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 nitrocellulose. 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 nitrocellulose; 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 perfomed 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).

Materials and Method

**Capture probe and ssDNA target**

The study used 4 sequences of oligonucleotide (27-28 base lengh) 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. Those 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).
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°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 was were 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.

### Table 1. Oligonucleotide sequence used in this study

| Sequence | Sequence 5’– 3’          |
|----------|---------------------------|
| D1       | CACCAGGGGAAGCTGTACCCTCGTGTTG | |
| D2       | GGTGAGTGAAAGCTGATCCTACTGG  | |
| D3       | GCACCTGAGGAAAGCTGATCCCTTGGCA | |
| D4       | AGCCAGGAGGAAGCTGATCTGTTGCTGG | |

| ssDNA    | Biotin-GGTCCTCTCTAACCTTGCTAACCACCCGGTTACAGCTTCCCCTTGTTGTTGGCCCGCGCTGTCTCCTGTACCC |

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

### Table 2. Oligonucleotide characteristics

| Capture Probe | Length (nt) | GC content (%) | Tm (°C) | Homology to ssDNA (%) | Length of 100% continue sequence complementary to ssDNA (nt) |
|---------------|-------------|----------------|---------|-----------------------|-------------------------------------------------------------|
| 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%
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 occur 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 unspecific base-pairing. It is known easier to form nucleobase binding at low temperature spontaneously, 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.

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

| Buffer       | Sample Concentration (pmol) | Color Signal | Test Line D1 | Test Line D2 | Test Line D3 | Test Line D4 |
|--------------|-----------------------------|--------------|--------------|--------------|--------------|--------------|
| 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                        | +/−          | +/−          | +/−          | +/−          | +/−          |
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). Glycerol 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

Glycerol 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 applied at room temperature..

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References

Anderson, M.L.M. and Young, B.D. (1985) Quantitative filter hybridization. In: *Nucleic Acid Hybridization, a Practical Approach*. Hames, B.D. and Higgins, S.J. (Eds.), IRL Press, Oxford, pp. 73-111.

Chang, C.T., Hain, T.C., Hutton, J.R. and Wetmur, J.G. (1974) Effect of microscopic and macroscopic viscosity on the rate of renaturation of DNA. Biopolymers 13 (1974) 1847-1858.

Dave, N. and Liu, J. (2010) Fast Molecular Beacon Hybridization in Organic Solvents with Improved Target Specificity, *J. Phys. Chem. B* 2010, 114, 15694–15699

Farrell-Jr, R.E. 2010. A Laboratory Guide for Isolation and Characterization In Practical Nucleic Acid Hybridization in RNA Methodologies (4th Edition), Academic Press, New York. p. 287

Gao, Y., Wolf, L.K. and Georgiadis, R. M. (2006). Secondary structure effects on DNA hybridization kinetics: a solution versus surface comparison. *Nucleic Acids Research*, 34 (11), doi: 10.1093/nar/gkl422, pp. 3370–3377.

Haslam, N.J., Whiteford, N.E., Weber, G., Prugel-Bennett, A., Essex, J.W., and Neylon, C. (2008). Optimal probe length varies for targets with high sequence variation: implications for probe library design for resequencing highly variable genes. *PLoS One*. 3(6):e2500

Jauset-Rubio, M., Svobodová, M., Mailal, M., Mc-Neil, C., Keegan, N., Saeed, A., Abbas, MN., El-Shahawi, MS., Bashammakh, AS, Alyoubi, AO., and O’Sullivan, CK., 2016. Ultrasensitive, rapid and inexpensive detection of DNA using paper based lateral flow assay. *Sci. Rep.* 6, 37732; doi: 10.1038/srep37732

Kusumawati, A., Tampubolon, I.D., Hendarta, N.Y., Salasie, S.I., Wanahari, T.A., Mappakaya, B.A. and Hartati, S. (2015). Use of reverse transcription loop-mediated isothermal amplification combined with lateral flow dipstick for an easy and rapid detection of Jembrana disease virus. Virusdisease., 26(3):189-95. doi: 10.1007/s13337-015-0277-5

Letowski, J., Broussseau,R., and Masson, L., 2004, Designing better probes: effect of probe size, mismatch position and number on hybridization in DNA oligonucleotide microarrays, *Journal of Microbiological Methods*, 57, pp. 269–278

Lyberopoulou, A., Efstathopoulos, E. P. and Gazouli. M. 2016. Chap. 6. Nanotechnology-Based Rapid Diagnostic Tests, in Proof and Concepts, in Rapid Diagnostic Tests and Technologies. Shailendra K. Saxena ed., InTechOpen, pp. 89-105

Reinhartz, A., Alajem, S., Samson, A. and Herzberg, M., (1993) A novel rapid hybridization technique: paper chromatography hybridization assay (PACHA), *Gene*, 136, 221-226
Sambrook, J. and Russel, D.W. 2001. *Molecular Cloning: A Laboratory Manual* vol.1. 3rd ed. Cold Spring Harbor Laboratory Press, New York, p. 10.2

Wang, X., Lim, HJ, and Son, A., 2014, Characterization of denaturation and renaturation of DNA for DNA hybridization, *Environ Health Toxicol.*, 29: e2014007

Weckx, S., Carlon, E., Vuyst, L.D. Hummelen, P. V. (2007) Thermodynamic behavior of short oligonucleotides in microarray hybridizations can be described using Gibbs free energy in a nearest-neighbor model. *J. Phys. Chem. B*, 111 (48), pp 13583–13590