Published in final edited form as:

Anal Chim Acta. 2012 December 13; 757: 69–74. doi:10.1016/j.aca.2012.10.052.

Importance of membrane selection in the development of immunochromatographic assays for low-molecular weight compounds

Ji-Ye Lee^a, Young Ah Kim^a, Mi Yeon Kim^a, Yong Tae Lee^b, Bruce D. Hammock^c, and Hye-Sung Lee^{a,*}

^aDepartment of Food Science and Nutrition, Kyungpook National University, Daegu 702-701, South Korea

^bDepartment of Molecular Life Science, Yeungnam University, Gyongsan 712-749, South Korea

^cDepartment of Entomology and Cancer Research Center, University of California, Davis, CA 95616, USA

Abstract

This study was performed to demonstrate the importance of selecting an appropriate membrane when developing immunochromatographic assays (ICAs) for the sensitive detection of low-molecular weight compounds. Based on our findings, we propose a theoretical basis for selecting such a membrane. When eluting the sample solution for the competitive ICA using colloidal gold label for low-molecular analytes, the degree of binding inhibition is proportional to the collision frequency between the antibody-colloidal gold (Ab–CG) and analyte before Ab–CG binding to the capture antigen and a higher concentration of pesticides around the Ab–CG leads to a greater degree of inhibition. Therefore, we propose that the relative migration speed of the analyte and Ab–CG on the test strip is critically important for selecting a membrane in the development of sensitive competitive ICAs. We developed a novel method to estimate such a relative migration speed. We demonstrated the applicability of this proposal by using it to select an appropriate membrane for the development of an ICA of the pesticide diazinon.

Keywords

Immunochromatographic assay; Low-molecular weight compounds; Membrane; Diazinon

1. Introduction

Immunochromatographic assays (ICAs) have recently begun to gain acceptance as simple, rapid, and inexpensive tools for detecting trace amounts of chemicals and biomolecules [1–3]. The key advantage of an ICA using colloidal gold as the label is that this technique involves only one step unlike other immunoassays that usually require three or four steps. ICAs specific for monovalent low-molecular weight compounds such as pesticides must be

competitive in contrast to ICAs for high-molecular compounds that usually involve a noncompetitive sandwich procedure [4–11]. Compared to the large number of other types of immunoassays developed for small molecules, the number of ICAs for small molecules is rather small. This is probably due to difficulties in establishing effective competition in a competitive ICA format. Pre-incubation of sample with Ab–CG before performing an ICA described in some papers appears to be evidence of such a difficulty [12–14].

In competitive ICAs, competition is between the migrating analyte and immobilized analyte hapten (capture antigen) for the binding to the migrating Ab–CG. The degree of inhibition of Ab–CG binding to the capture antigen would be proportional to the frequency of collision between Ab–CG and analyte before completion of Ab–CG binding to the capture antigen. Meanwhile, the collision frequency would depend on the concentration of analyte around the migrating Ab–CG and the time required for Ab–CG to reach the capture antigen. Concentration of the analyte around the migrating Ab–CG, in turn, would depend on the relative migration speed of the analyte and Ab–CG on the test strip. Therefore, we suggest in the present study that the relative migration speed of the two migrating substances is critically important for sensitive detection by a competitive ICA. We also propose that a suitable relative migration speed of the two migrating substances depends on the type of ICA. We previously discussed the topic of suitable relative migration speed in lateral ICAs [10,11] and we further explore this issue in the current study. Here, we also present a discussion about suitable relative migration speeds for dipstick type ICA.

The proposal on suitable relative migration speeds for two types of ICAs was also tested in this study by using the proposal to select an appropriate membrane for detecting the organophosphorus pesticide diazinon. Using the selected membrane and a monoclonal antibody to diazinon, an ICA for the pesticide was developed and validated.

2. Experimental

2.1. Materials and chemicals

Pesticides including diazinon were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Gold (III) chloride trihydrate, sodium citrate, BSA, ovalbumin (OVA), polyethylene sorbitan monolaurate (Tween 20), phosphate buffered saline (PBS), indophenyl acetate (IPA), and anti-mouse IgG were purchased from Sigma (St. Louis, USA). Cellulose (Rapid 24) for sample pad and NC membranes (nitrocellulose 8.0, Immunopore RP, FP and SP) for sample pad were obtained from Whatman (Maidstone, UK). Cellulose (Millipore SA3H645H9) for the absorption pad was acquired from Millipore (Billerica, MA).

2.2. Capture antigen and antibody

The monoclonal anti-diazinon antibody and capture antigen (diazinon hapten-OVA) used for this study were ones previously prepared in the laboratory of one of the authors (Y.T.L.) [15].

2.3. Preparation of the Ab–CG complexes

Colloidal gold was prepared using the method developed by Frens [16]. The procedure was as follows. Fifty mL of 0.01% tetra-chloroauric acid solution was boiled and 1 mL of 1% sodium citrate solution was added under constant stirring. Stirring was continued until the color changes from purple to reddish-orange and, then, the solution was cooled. The cooled solution was adjusted to pH 9.0 with 0.1 M K₂CO₃. Conjugation of the anti-diazinon antibody to colloidal gold was carried out according to the method by Roth [17]. The optimal ratio of the antibody to colloidal gold was determined using the procedure by Beasley [18]. Before conjugation, the optimal concentration of antibody for conjugation was determined. One milliliter of colloidal gold solution was distributed into each of a series of vial. The antibody solution (0–15 μ L) was added to each vial. The vials were shaken for 1 min and then incubated for 5 min. One hundred microliter of 10% NaCl was added to each vial. After 1 min, a minimum amount of antibody was evaluated by the color change from reddish to blue. If a vial contained a minimum amount of the antibody, the color of the colloidal gold particles was not changed. For conjugation, 130 μ L of antibody (1.0 mg mL⁻¹) was added drop by drop to 10 mL colloidal gold solution and incubated at room temperature under stirring. After 30 min, 1.1 mL of 1% BSA was added to block residual surfaces of the colloidal gold and the mixture was incubated for 30 min at room temperature. Then, the mixture was centrifuged (12,000 rpm) at 4 °C for 20 min and the supernatant was discarded. The pellets were suspended in 1 mL of 10 mM PBS buffer (pH 7.4) and were stored at 4 °C until being used.

2.4. Evaluating the relative mobility of the pesticide and Ab–CG on the membranes

We previously developed a method to measure the relative migration speed of two migrating substances, pesticide and Ab–CG, on a membrane [10,11]. This method was further tested in the present study by using it to select a suitable membrane for an ICA specific for diazinon. The membranes tested were four NC membranes, nitrocellulose 8.0, and Immunopore SP, FP, and RP from Whatman. Since diazinon is colorless, a colored substitute compound similar to diazinon in polarity was sought. We chose IPA, which is relatively non-polar and dark brown in color [10].

Relative mobility of the two migrating substances was measured as follows. Ab–CG (1 μ L) was pipetted onto the upper end of the sample pad of the lateral or dipstick test strip (see Section 2.5 below). The eluted solutions were 5, 10, and 20% methanol-PBST (PBS with Tween 20) containing 10 mM IPA. Flow times were measured when the IPA and Ab–CG reached the test line. Membranes blocked with either 0.1% or 1% BSA for 30 s and 30 min were also tested.

2.5. Preparation of ICA test strips and ICA procedure

Structures of the test strips used for the lateral and dipstick ICAs are shown in Figs. 1 and 2, respectively. A thin test line was formed on the membrane by applying the capture antigen in PBS using a Hamilton syringe. The control line was similarly formed by applying anti-mouse IgG in PBS above the capture antigen. The membrane with the test and control lines was pasted onto the central part of the plastic backing sheet using double-sided adhesive tape. The cellulose membrane used as a sample pad was pasted onto the lower part of the

backing sheet over-lapping the other attached membrane with a 2-mm width. The cellulose membrane used as an absorbent pad was similarly pasted onto the upper part of the backing sheet.

The assembled plate was cut lengthwise into square strips 0.4 cm × 5.9 cm in size. Ab–CG was applied to the upper end of the sample pad. Two types of ICA formats were used: lateral and dipstick. For the lateral assay, the membrane was soaked by the dropping sample solution onto the test strip placed horizontally on a flat surface. For the dipstick assay, the membrane was soaked by dipping the test strip into the sample solution. The lateral type strip was used either without further manipulation or as an in-housed format for which the test strip was mounted into a cassette with a well for sample application and a window for detection. The assay was performed using 80 µL of serial dilutions of the diazinon standards or samples spiked with diazinon.

2.6. Optimization of the ICA test strip

The Tween 20 concentration in the eluting solvent (20% methanol-PBST) was optimized by testing concentrations of 0, 0.05, 0.075, and 0.1%. The position of the capture antigen (1 µL cm⁻¹) was optimized by testing positions 0.7, 1.0, and 1.3 cm from the bottom of the signal pad. The concentrations of the capture antigen solution tested were 0.5, 1.0, 1.5, and 2.0 mg mL⁻¹.

2.7. Performance of the ICA test strip for detecting diazinon in the standard solutions

Performance of the optimized ICA was tested by using it to detect diazinon in standard solutions containing 10²–10⁻³ µg mL⁻¹ of the pesticide. The minimal detection limit was defined as the lowest pesticide concentration that produced a “positive” score in five tests by six examiners.

2.8. Performance of the ICA test strip for detecting diazinon in agricultural samples

Diazinon was extracted from the spiked samples using a previously described protocol [10,11]. The extract was analyzed with the lateral ICA strip.

2.9. Determination of cross-reactivity in the ICA

Cross-reactivity in the two types of ICAs developed by us was tested using several organophosphorus pesticides, a metabolite of organophosphorus pesticides, and a carbamate pesticide. The level of cross-reactivity was visually estimated based on “positive” or “negative” results.

3. Results and discussion

3.1. Theoretical considerations for the desirable relative mobility of the pesticide and Ab–CG on the membranes

An ICA for detecting low-molecular weight compounds requires competition between an analyte and its competitor. In currently used ICAs, this competition is between the migrating analyte and an immobilized capture antigen for binding sites on the migrating Ab–CG. Intensity of the red pigmentation of the Ab–CG captured at the test line is inversely

proportional to the degree of inhibition of Ab–CG binding to the immobilized capture antigen by the analyte. In turn, the degree Ab–CG binding inhibition is proportional to the collision frequency between the Ab–CG and analyte before Ab–CG binds to the capture antigen. Theoretically, the collision frequency would depend on the concentration of the pesticide around the migrating Ab–CG, and the time required for the Ab–CG complex to reach the capture antigen.

Meanwhile, concentration of the pesticide around the Ab–CG would depend on the relative migration speed of the pesticide and Ab–CG on the test strip. In lateral flow ICAs where the sample is applied once for all, the analyte must go ahead of the Ab–CG, but not too far from the Ab–CG to achieve maximum overlap of the two substances. The migration speed of the analyte should therefore be slightly higher than that of the Ab–CG. For dipstick ICAs where the sample is continuously applied, the situation is drastically different. The analyte must pass the Ab–CG, and increased analyte speed produces greater inhibition. Longer migration times due to slower migration speeds lead to a higher degree of inhibition. However, since slow migration speed of the Ab–CG requires an undesirable long assay performance time, compromise is needed when identifying an appropriate Ab–CG migration speed.

A preliminary theoretical considerations similar to those in the above paragraph were used in our previous studies to select a suitable membrane for lateral ICA of the pesticides chlopyrifos and EPN [10,11]. Among the various membranes, which are currently used as solid supports for the adsorption or filtration of biomolecules, NC membrane was the most suitable. NC membranes have been commonly used as a signal pad in most ICA test strips because they are associated with a sufficient flow rate due to their large pore sizes [19]. Companies manufacturing these membranes usually offer several types of NC membranes. We decided to test our proposal presented above by applying it to the selection of the most appropriate NC membranes among the four NC membranes produced by Whatman.

3.2. Estimation of the relative pesticide and Ab–CG mobility on the membranes

Diazinon is colorless and not visible on the test strip. Therefore, IPA with a dark brown color and weak polarity was used as a substitute for diazinon. Results of the experiment carried out to estimate the mobility of IPA and Ab–CG on the lateral and dipstick test strips are presented in Tables 1 and 2, respectively. The two reagents migrated with tailing, so their mobility in lateral elution was calculated as an interval of the flow time (the period required for the front and end part of the reagent spot to reach the test line). For the dipstick test, mobility was calculated as the interval of the flow time when the adsorption of IPA on the test line started and ended.

Among the four types of NC membranes tested with lateral elution, the migration speed of IPA and Ab–CG were too fast on the nitrocellulose 8.0 and Immunopore RP membranes (Table 1). The assay performance time of ICA is approximately the time for Ab–CG to reach the test line; thus, it was less than 2 min with the two membranes. These two membranes were therefore not suitable for the ICA. The two remaining membranes, Immunopore SP and FP, produced suitable migration speeds, but the migration speed of IPA was much slower compared to that of Ab–CG, which was undesirable. However, considerable overlap of the migration times of the two reagents was observed when eluted

with 20% methanol-PBST. The two membranes were therefore expected to be suitable for ICA if 20% methanol-PBST were used as the solvent. Of the two membranes, Immunopore FP was chosen as more suitable on the basis of the better sensitivity when they were used for ICA of diazinon.

Similar to the lateral elution test, the dipstick elution test showed that the migration speeds of Ab-CG and IPA were too fast (less than 1 min) on nitrocellulose 8.0 and Immunopore RP membranes (Table 2). These two membranes were thus considered unsuitable for ICAs. The two remaining membranes, Immunopore SP and FP, produced suitable migration speeds, but no overlap of migration times of the two reagents was observed. However, the migration times of the two reagents were closest when eluted with 20% methanol-PBST, the solvent chosen for the dipstick ICA. Despite a lack of overlapping migration times, the two membranes showed inhibition when 20% methanol-PBST was used as the solvent. Between the two membranes, Immunopore FP was associated with greater sensitivity when used for the diazinon-specific ICA.

Blocking of membrane surface with a protein may influence the mobility of substances on the membranes. It was found that blocking the four NC membranes increased the migration speeds of both Ab-CG and IPA, but the increase in IPA migration speed was less than that of Ab-CG (data not shown). These findings demonstrate that membrane blocking was not a useful method for improving the relative migration speeds of the two substances.

3.3. Optimization of the ICA format

Use of the test strip made from Immunopore FP membrane was further optimized by testing different concentrations of Tween 20 in the elution solvent, and evaluating the effects of position and concentration of the capture antigen. A 0.05% concentration of Tween 20 was the most suitable for both the lateral and dip-stick assays. Optimal positions of the capture antigen were 0.7 cm and 1.0 cm from the bottom of the membrane for the lateral and dipstick assays, respectively. The best capture antigen concentration for both assays was 1 mg mL⁻¹.

3.4. Performance of ICA test strip

Results of the lateral and dipstick ICAs using the optimized test strip and diazinon standard solutions are presented in Figs. 3 and 4, respectively. In both types of assays, a negative control without diazinon produced a clear red color both at the test line and control line. In both types of assays, diazinon at concentrations above 10⁻² µg mL⁻¹ was colorless at the test line, but red color development was observed at the control line. A diazinon concentration of 10⁻³ µg mL⁻¹ produced an obvious difference compared to the negative control. Therefore, the visual detection limit of diazinon for both the lateral and dipstick ICAs was considered to be 10⁻³ µg mL⁻¹.

Results of ICA evaluating rice samples spiked with diazinon are shown in Fig. 5. The diazinon detection limit was 10⁻² µg mL⁻¹. Similar results were obtained from the ICA assessing lettuce samples spiked with diazinon (Fig. 6). Matrix interference was relatively insignificant. Cross-reactivities of several organophosphorus pesticides, a carbamate pesticide, and a diazinon metabolite observed in the lateral ICA are presented in Table 3.

Cross-reactivities in the dip-stick ICA was similar. Interference with the assays was negligible in all cases.

4. Conclusion

The purpose of this study was to demonstrate the importance of selecting an appropriate membrane when developing ICAs for detecting low-molecular weight compounds. Conditions that a membrane must have for sensitive detection were explored theoretically. During elution of the sample solution in competitive ICAs for low-molecular analytes using colloidal gold label, the migrating analyte and immobilized capture antigen compete for binding sites in the migrating Ab–CG complex. We noted the fact that the degree of binding inhibition is proportional to the collision frequency between the Ab–CG complex and analyte. Thus, higher concentrations of pesticides around the Ab–CG would lead to a greater degree of inhibition. We therefore proposed that relative migration speeds of the analyte and Ab–CG on the test strip is critically important for membrane selection during the development of competitive ICAs. For lateral flow ICAs, we concluded that a membrane on which the migration speed of the analyte is slightly higher than that of the Ab–CG is desirable. For dipstick ICAs, we determined that a membrane on which the migration speed of the analyte is much higher than that of the Ab–CG is most appropriate. We tested these hypotheses by using them to select the most suitable membrane for ICAs specific for detecting the pesticide diazinon. These assays were both sensitive and selective, thereby demonstrating the usefulness of our proposed criteria developed in this study.

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean Government (no. 2010-0003669). Partial support was provided by NIH Counter Act Program U54 NS079202-01.

References

1. Wong, RC.; Tse, HY., editors. Lateral Flow Immunoassay. Humana Press; New York: 2009.
2. Rasooly, A.; Herold, K., editors. Biosensors and Biodetection: Methods and Protocols. Vol. 2. Humana Press; New York: 2010. p. 169-254.
3. Ngom B, Guo Y, Wang X, Bi D. Anal Bioanal Chem. 2010; 397:1112–1135.
4. Shim WB, Yang ZY, Kim JY, Choi JG, Je JH, Kang SJ, Kolosova AY, Eremin SA, Chung DH. J Agric Food Chem. 2006; 54:9728–9734. [PubMed: 17177493]
5. Kaur J, Vikas Singh K, Boro R, Thampi KR, Raje M, Varshney GC, Raman Suri C. Environ Sci Technol. 2007; 41:5028–5036. [PubMed: 17711219]
6. Zhu J, Chen W, Lu Y, Cheng G. Environ Pollut. 2008; 156:136–142. [PubMed: 18255209]
7. Guo YR, Liu SY, Gui WJ, Zhu GN. Anal Biochem. 2009; 389:32–39. [PubMed: 19303861]
8. Kranthi KR, Davis M, Mayee CD, Russell DA, Shukla RM, Satija U, Kshirsagar M, Shiware D, Kranthi S. Crop Protect. 2009; 28:428–434.
9. Hua X, Qian G, Yang J, Hu B, Fan J, Qin N, Li G, Wang Y, Liu F. Biosens Bioelectron. 2010; 26:189–194. [PubMed: 20598518]
10. Kim YA, Lee EH, Kim KO, Lee YT, Hammock BD, Lee HS. Anal Chim Acta. 2011; 693:106–113. [PubMed: 21504817]
11. Lee, E-H.; Kim, YA.; Lee, YT.; Hammock, BD.; Lee, H-S.; Agric, J. Immunol. <http://dx.doi.org/10.1080/09540105.2012.661703>
12. Wang S, Zhang C, Wang J, Zhang Y. Anal Chim Acta. 2005; 546:161–166.

13. Zhang C, Zhang Y, Wang SJ. *J Agric Food Chem*. 2006; 54:2502–2507. [PubMed: 16569035]
14. Li K, Liu L, Chu X. *Anal Sci*. 2007; 23:1281–1284. [PubMed: 17998745]
15. Lee EK, Kim YJ, Park WC, Chung TW, Lee YT. *Anal Chim Acta*. 2005; 530:143–153.
16. Frens G. *Nat Phys Sci*. 1973; 241:20–22.
17. Roth J. *Histochem J*. 1982; 14:791–801. [PubMed: 7129958]
18. Beasley, JE. *Colloidal Gold: A New Perspective for Cytochemical Marking*. Oxford University Press; Kent, UK: 1989. p. 1-14.
19. Mansfield, MA. *Lateral Flow Immunoassay*. Wong, RC.; Tse, HY., editors. Humana Press; New York: 2009. p. 96

HIGHLIGHTS

- Membrane selection for competitive immunochromatographic assay (ICA) is discussed.
- Relative migration speed of analyte and antibody-colloidal gold (Ab–CG) is important.
- For lateral flow ICAs, analyte moving slightly faster than Ab–CG is desirable.
- For dipstick ICAs, analyte moving much faster than Ab–CG is desirable.
- The proposal was useful in membrane selection for ICA of the pesticide diazinon.

Fig. 1.
Schematics of the lateral ICA strip.

Fig. 2.
Schematics of the dipstick ICA strip.

Fig. 3.

Results of the lateral ICA with diazinon standards. The structure of the ICA strip used is illustrated in Fig. 1.

Fig. 4. Results of the dipstick ICA with diazinon standards. The structure of the ICA strip used is illustrated in Fig. 2.

Fig. 5. Results of the lateral ICA for detecting diazinon in rice samples. The structure of the ICA strip used is illustrated in Fig. 1.

Fig. 6. Results of the lateral type ICA for detecting diazinon in lettuce samples. The structure of the ICA strip used is illustrated in Fig. 1.

Table 1

The mobility^a of Ab-CG and IPA on 4 types of membranes in lateral type elution.^b

	Nitrocellulose 8.0				Immunopore SP				Immunopore FP				Immunopore RP			
	IPA	Ab-CG	Solvent		IPA	Ab-CG	Solvent		IPA	Ab-CG	Solvent		IPA	Ab-CG	Solvent	
5% Methanol-PBST	45–67	16–23	9		277–531	88–162	18		361–537	144–208	16		121–219	59–86	12	
10% Methanol-PBST	39–61	16–25	9		268–504	113–174	18		314–505	150–231	16		103–209	64–95	12	
20% Methanol-PBST	39–57	24–43	12		147–328	131–207	18		206–337	162–243	17		100–160	83–109	13	

^aThe range of the time (s) from the beginning of the color development or wetting at the test line to the end.

^bThe lateral type strip shown in Fig. 1 was used. The solutions developed were 5, 10 and 20% methanol-PBST containing 10 mM IPA.

Table 2

The mobility^a of Ab-CG and IPA on 4 types of NC membranes in dipstick type elution.^b

	Nitrocellulose 8.0			Immunopore SP			Immunopore FP			Immunopore RP		
	IPA	Ab-CG	Solvent	IPA	Ab-CG	Solvent	IPA	Ab-CG	Solvent	IPA	Ab-CG	Solvent
5% Methanol-PBST	60-72	22-27	18	221-463	59-100	20	301-522	86-107	17	114-185	40-50	14
10% Methanol-PBST	60-72	21-26	18	194-445	72-106	22	298-492	94-120	19	120-178	42-60	15
20% Methanol-PBST	53-63	24-35	19	144-432	105-143	25	229-454	128-170	22	97-137	53-67	16

^aThe range of the time (s) from the beginning of the color development or wetting at the test line to the end.

^bThe dipstick type strip shown in Fig. 2 was used. The solution was developed by dipping the strip in 5, 10 or 20% methanol-PBST containing 10 mM IPA.

Table 3
Cross-reactivity^a of compounds structurally related to diazinon in lateral type ICA.

Compound	Concentration ($\mu\text{g mL}^{-1}$)									
	10 ²	10 ¹	10 ⁰	10 ⁻¹	10 ⁻²	10 ⁻³	0			
Diazinon	Control line	a+	+	+	+	+	+	+	+	+
	Test line	-	-	-	-	-	-	±	±	+
Pyrimiphos-ethyl	Control line	+	+	+	+	+	+	+	+	+
	Test line	+	+	+	+	+	+	+	+	+
Parathion-ethyl	Control line	+	+	+	+	+	+	+	+	+
	Test line	+	+	+	+	+	+	+	+	+
Bromophos-ethyl	Control line	+	+	+	+	+	+	+	+	+
	Test line	+	+	+	+	+	+	+	+	+
EPN	Control line	+	+	+	+	+	+	+	+	+
	Test line	+	+	+	+	+	+	+	+	+
Isofenphos	Control line	+	+	+	+	+	+	+	+	+
	Test line	+	+	+	+	+	+	+	+	+
Bromophos-methyl	Control line	+	+	+	+	+	+	+	+	+
	Test line	+	+	+	+	+	+	+	+	+
Fenthion	Control line	+	+	+	+	+	+	+	+	+
	Test line	+	+	+	+	+	+	+	+	+
Parathion-methyl	Control line	+	+	+	+	+	+	+	+	+
	Test line	+	+	+	+	+	+	+	+	+
2-Isopropyl-6-methyl-4-pyrimidinol	Control line	+	+	+	+	+	+	+	+	+
	Test line	+	+	+	+	+	+	+	+	+
Dichlofenthion	Control line	+	+	+	+	+	+	+	+	+
	Test line	+	+	+	+	+	+	+	+	+
Azinphos-ethyl	Control line	+	+	+	+	+	+	+	+	+
	Test line	+	+	+	+	+	+	+	+	+

^a +, obvious red band; ±, faint red band; -, no red band.