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Antibody Labeling Kits vs Conventional Labeling: A Comparative Study in Immunofluorescence of Cell Cultures

Antikor Etiketleme Kitleri ile Geleneksel Etiketleme: Hücre Kültürlerinde Karşılaştırmalı İmmünofloresans Bir Çalışma

Kubilay Doğan Kılıç[1,2,3] Büşra Horuz[1] Ayşegül Taşkıran[1]

[1] Ege University, Faculty of Medicine, Department of Histology and Embryology, İzmir, Türkiye

[2] Helmholtz Center, Institute for Tissue Engineering and Regenerative Medicine (iTERM), Munich, Germany

[3] Leibniz Institute for Evolution and Biodiversity Science, Museum für Naturkunde, Berlin, Germany

ABSTRACT

Aim: This study aims to evaluate the comparative performance of antibody labeling kits and conventional antibody labeling methods regarding efficiency, accuracy, and practical usability in immunofluorescence applications on cell cultures.

Materials and Methods: Both labeling methods were applied to identical cell culture samples. Labeling efficiency, fluorescence intensity, and antibody specificity were assessed using quantitative fluorescence microscopy. Practical aspects such as ease of use, time efficiency, and cost were also systematically evaluated.

Results: Antibody labeling kits significantly reduced preparation time while increasing labeling efficiency, achieving comparable results to conventional methods in fluorescence intensity and antibody specificity. In sum, it is reported that it is higher ease of use for Antibody Labeling Kits.

Conclusion: Antibody labeling kits offer a reliable and user-friendly alternative for immunofluorescence studies. By enhancing labeling efficiency and simplifying operational processes, these kits can accelerate research workflows and improve experimental outcomes in cell-based assays.

Keywords: Antibody binding stability, quantitative fluorescence microscopy, multicolor labeling, biomolecular imaging, efficiency.

ÖZ

Amaç: *Bu çalışma, antikor işaretleme kitleri ile geleneksel antikor işaretleme yöntemlerinin immün floresans uygulamalarında hücre kültürleri üzerindeki verimlilik, doğruluk ve pratik kullanılabilirlik açısından karşılaştırmalı performansını değerlendirmeyi amaçlamaktadır.*

Gereç ve Yöntem: *Çalışmada her iki işaretleme yöntemi, aynı hücre kültürü örneklerine uygulanmıştır. İşaretleme verimliliği, floresans yoğunluğu ve antikor özgüllüğü, nicel floresans mikroskopisi ile değerlendirilmiştir. Ayrıca kullanım kolaylığı, zaman verimliliği ve maliyet gibi pratik unsurlar incelenmiştir.*

Bulgular: *Antikor işaretleme kitleri, hazırlık süresini önemli ölçüde azaltırken işaretleme verimliliğini arttırmış ve floresans yoğunluğu ile antikor özgüllüğü açısından geleneksel yöntemlere eşdeğer sonuçlar sağlamıştır. Bütün değişkenler bir arada değerlendirildiğinde, antikor İşaretleme Kitlerinin kullanım kolaylığını daha yüksek olarak bildirmiştir.*

Corresponding author: Kubilay Doğan Kılıç
Ege University, Faculty of Medicine, Department of Histology and Embryology, İzmir, Türkiye
E-mail: *kubilay.dogan.kilic@ege.edu.tr*
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Sonuç: *Antikor işaretleme kitleri, immün floresans çalışmaları için güvenilir ve kullanıcı dostu bir alternatif sunmaktadır. Etiketleme verimliliğini artırmaları ve operasyonel süreçleri basitleştirmeleri, bu kitlerin araştırma süreçlerini hızlandırarak hücre temelli deneylerde daha iyi sonuçlar elde edilmesini sağlayabileceğini göstermektedir.*

Anahtar Sözcükler: *Antikor bağlanma stabilitesi, nicel floresans mikroskopisi, çok renkli etiketleme, biyomoleküler görüntüleme, verimlilik*

INTRODUCTION

Antibody labeling is a fundamental technique in immunofluorescence (IF), enabling the visualization of specific antigens within biological samples. Traditional methods, such as direct conjugation, where dyes are chemically attached to primary antibodies (1) and indirect conjugation, which utilizes secondary antibodies to bind to primary antibodies, have been foundational tools for decades (2-3) (Figure-1). Despite their robustness, these methods often require meticulous optimization to address challenges like non-specific binding and high background fluorescence.

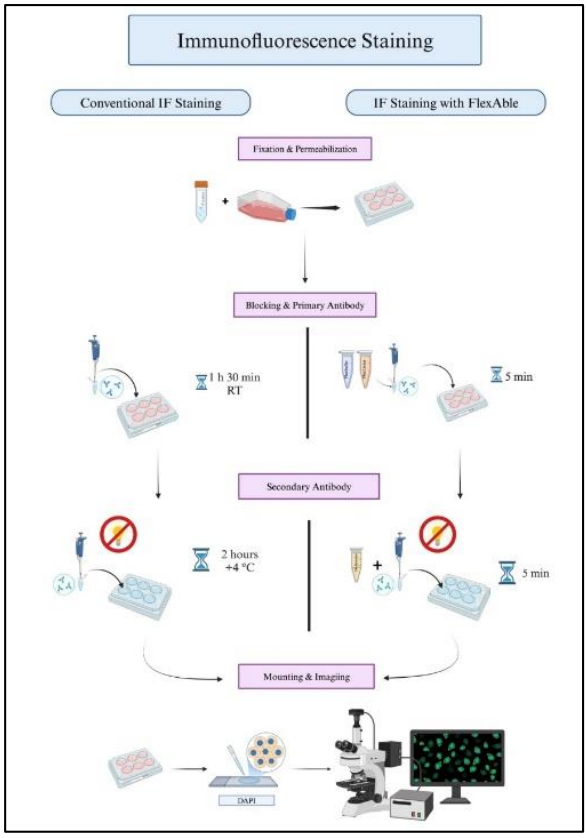

Figure-1. IF staining protocols. RT: Room temperature. Created in https://BioRender.com

Recent advancements in the field have introduced kit-based solutions that aim to simplify and enhance the efficiency of antibody labeling. Examples include the FlexAble Antibody Labeling Kits (Proteintech, NJ, USA) (4), Lightning-Link® (Abcam, CA, USA) (5-7), and EasyLink Labeling Kits (Abcam, CA, USA) (8-11). These kits streamline the labeling process, reducing hands-on time and the complexities associated with traditional conjugation methods.

The accuracy and reliability of immunofluorescence heavily depend on the quality of antibody labeling. Efficient and precise labeling is critical for achieving high specificity and sensitivity, which are essential for accurate molecular interaction studies and protein localization analyses. Conversely, inefficient labeling can lead to weak fluorescence signals and significant data misinterpretation, ultimately hindering scientific progress. As biological research questions grow increasingly complex, there is an urgent demand for reliable, straightforward labeling techniques that align with the needs of fast-paced research environments. Kit-based solutions, such as FlexAble kits, represent a promising innovation in this area, offering potential benefits like reduced variability and increased consistency. Their impact on accelerating biomedical research and improving data reliability makes their evaluation essential.

This study aims to compare the performance, convenience, and cost-effectiveness of Antibody Labeling Kits and traditional labeling methods. Key objectives include evaluating labeling efficiency, fluorescence intensity, and antibody specificity using quantitative microscopy. Practical usability, time efficiency, and cost-effectiveness are also assessed to determine the optimal approach for immunofluorescence applications. The findings aim to guide researchers in selecting the most reliable and efficient tools for their studies.

MATERIALS AND METHODS

Subjects/Samples:

In the study, a type of human prostate cancer cell line DU145 (purchased from the American Type Cell Culture Collection-ATCC, HTB-81 ™, Manassas, VA) was used. Two different staining were performed on the cells for IF staining. First, the FlexAble CoraLite® Plus 647 Antibody

Labeling Kit for Rabbit IgG was used. For this, the FlexLinker, FlexBuffer and FlexQuencher buffers in the kit were used and the study was carried out using caspase-3 (Santa Cruz Biotechnology, INC.caspase-3 (31A1067): sc-56053) as the primary antibody and Alexa Fluor® 488 AffiniPure™ Rabbit Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc. 315-545-003) as the secondary antibody (Table-1).

Labeling Techniques:

While standard procedures were applied for conventional IF staining application, the staining protocol published by Proteintech was followed in our IF study with FlexAble Kit (4).

IF staining protocol performed with FlexAble Kit (4):

I. DU145 cell line (were purchased from the American Type Cell Culture Collection-ATCC, HTB-81 ™, Manassas, VA) cells were fixed with 4% paraformaldehyde (PFA) for 30 minutes and then washed with phosphate buffered saline (PBS) (BioShop Canada Inc. PBS 404).
II. For the permeabilization step, cells were kept in Triton X-100 (X100, Sigma-Aldrich) for 10 minutes and then washed again with PBS.
III. After these procedures, cells were treated with bovine serum albumin (BSA) (BSA-15, Capricorn) for 1 hour for blocking.
IV. Then, to equalize the total volume to 8 µL for staining with the components in the kit; 1 µL of FlexLinker was added to 0.5 µL of caspase-3 (Santa Cruz Biotechnology, caspase-3 (31A1067): sc-56053) used as primary antibody, 3.8 µL of FlexBuffer was added and incubated in the dark for 5 minutes.
V. Then, the secondary antibody Alexa Fluor® 488 AffiniPure™ Rabbit Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc. 315-545-003) and 2 µL of FlexQuencher were added and kept in the dark for 5 minutes.
VI. Cover slips with cells for mounting were placed on the slides and the slides were mounted using 4′,6-diamidino-2-phenylindole (DAPI) (UltraCruz® Aqueous Mounting Medium with DAPI: sc-24941).
VII. The slides were visualized under a fluorescence microscope for detection of findings (Figure-2).

The total processing time was approximately 1 hour (Table-2).

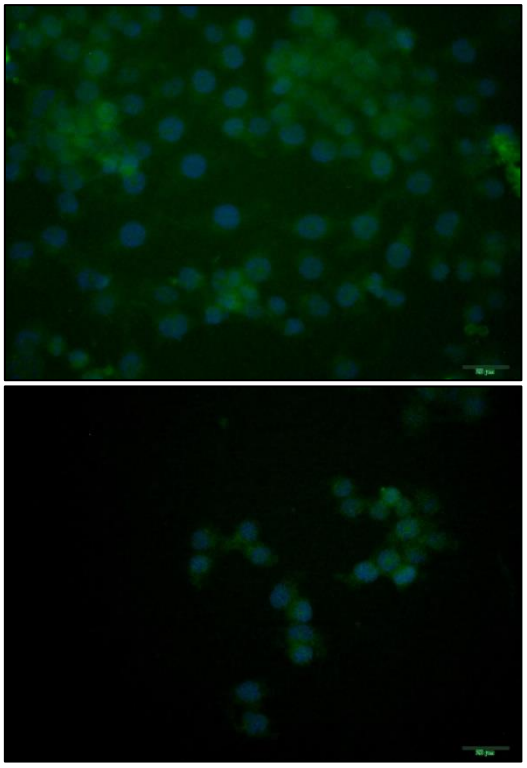

Figure-2. IF staining with FlexAble Labeling Kit on DU 145 cell line.

Table-1. Material list.

MATERIAL LIST	PRODUCT BRAND	CATALOG NO
Du 145 Cell Line	American Type Cell Culture Collection-ATCC	HTB-81
FlexAble Labeling Kit	Proteintech	KFA003
Caspase-3	Santa Cruz Biotechnology	sc-56053
Alexa Fluor® 488 AffiniPure™ Rabbit Anti-Mouse IgG (H+L)	Jackson ImmunoResearch Laboratories, Inc.	315-545-003
Pbs	BioShop Canada Inc.	PBS404
Triton X-100	Sigma-Aldrich	X100
BSA	Capricorn	BSA-15
DAPI	UltraCruz®	sc-24941

Then, the same cell line standard IF protocol was used for comparison. Conventional IF staining protocol applied in this study:

I. DU145 cell line cells in 6 well-plates were fixed with 4% PFA for 30 minutes and then washed with PBS (BioShop Canada Inc. PBS404).

II. cells were treated with permeabilization buffer Triton X-100 (X100, Sigma-Aldrich) for approximately 15 minutes and then washed again with PBS.

III. Then, cells were treated with BSA (BSA-15, Capricorn), which was used as a suitable solution for blocking. Then, cells were washed again with PBS and prepared for primary antibody application.

IV. Caspase-3 (Santa Cruz Biotechnology, caspase-3 (31A1067): sc-56053) was used as the primary antibody. After the primary antibody was applied to the cells overnight at fridge, each coverslip was washed 3 times with PBS for 3 minutes and prepared for secondary antibody application.

V. The secondary antibody used in the cells was Alexa Fluor® 488 AffiniPure™ Rabbit Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc. 315-545-003). For effective work of secondary antibodies, antibody application was performed in a dark work area for 1 hour or 1 hour and 30 minutes, and then the cells were washed 3 times with PBS.

VI. For the mounting stage, cells in 6 well-plates were placed on slides and mounted with DAPI (UltraCruz® Aqueous Mounting Medium containing DAPI: sc-24941).

VII. The slides were examined under a fluorescence microscope to visualize the findings (Figure-3).

The total procedure time was approximately 1 day (Table-2).

Following the completion of both IF studies, all slides were subjected to examination under a fluorescence microscope (Olympus/BX51) and subsequent analysis of the images using dedicated software (Olympus CellSens Software and Fiji by ImageJ).

Analytical Methods:

The performance of antibody labeling methods was evaluated using a comprehensive analytical approach. Labeling efficiency, fluorescence intensity, antibody specificity, and practical usability were systematically assessed to provide a robust comparison between the FlexAble Kit and conventional immunofluorescence staining methods.

Labeling efficiency was quantified using fluorescence microscopy (Olympus/BX51) with the CellSense software. Fluorescence intensity values were extracted from images and processed using ImageJ. Regions of interest (ROIs) were selected to calculate the mean fluorescence intensity (MFI), and each experiment was conducted in triplicate to ensure the reproducibility of results.

Antibody specificity was determined by analyzing the colocalization of labeled antibodies with known cellular markers. Pearson's correlation coefficient and Manders overlap coefficient were calculated using the Coloc2 plugin in ImageJ, providing quantitative metrics for the spatial overlap between fluorescence signals and target antigens (Figure-4).

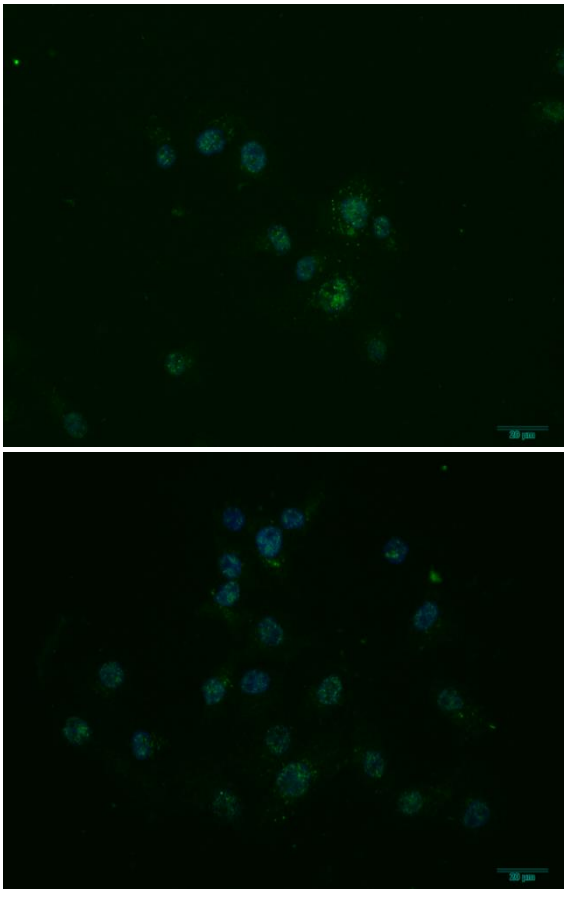

Figure-3. Conventional IF. DU 145 cell line.

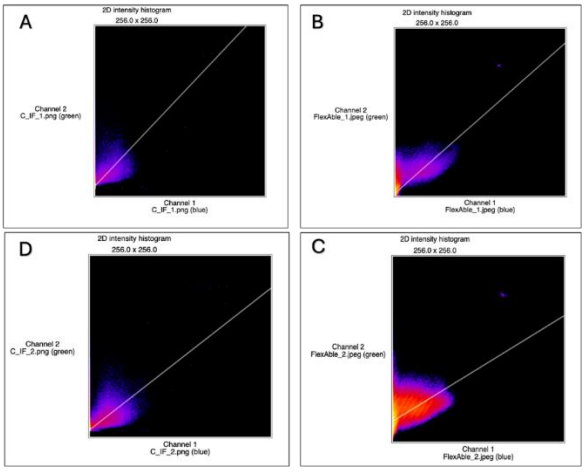

Figure-4. Comparison of histogram plots of IF staining and Coloc2 analyses. In the conventional method, the high Manders tM1 values indicate that Channel 2 shows a strong colocalization with Channel 1, but Channel 1 makes a more limited contribution to Channel 2 due to the low tM2 values. In contrast, FlexAble selectively reduced colocalization, allowing more specific analyses. FlexAble IF 2 showed a significant increase in the tM2 value, indicating that mutual colocalization improved. When the Pearson correlation values were examined, it was observed that FlexAble kept the colocalization lower in the suprathreshold regions while maintaining the correlation in the subthreshold regions. These findings indicate that FlexAble methods can be used as a more sensitive tool in cases where selective reduction or increase of colocalization is required. Channel 1(Ch1): Green, Channel 2 (Ch2): Blue. Manders' tM1 (Above auto threshold of Ch2), Manders' tM2 (Above auto threshold of Ch1).

These analyses ensured the accuracy and precision of antibody labeling. Time and cost efficiency metrics were also examined. The total time required for staining and the amount of reagents used were compared between the two methods. Procedural steps, such as blocking and secondary antibody applications, were simplified in the FlexAble Kit protocol, significantly reducing overall workflow complexity. Comparative results are summarized in Table-2.

Quantitative data were analyzed using statistical software SPSS (IBM SPSS Statistics for Windows, Version 25.0 (Armonk, NY: IBM Corp.IBM)). The Shapiro-Wilk test was employed to verify the normality of data distribution. Comparisons of labeling efficiency, fluorescence intensity, and signal stability between methods were performed using the Student's t-test or ANOVA as appropriate, with a significance level set at $p < 0.05$. All experiments were carried out in triplicate to ensure reproducibility of the results. Data are presented as mean ± standard deviation and graphical representations were created using GraphPad Prism 5.0 to visually depict the findings.

Comparative visualizations of efficiency, stability, and workflow simplification were created to illustrate the advantages of the FlexAble Kit (Table-2) (Figure-1). These visualizations highlight its potential to streamline research workflows and enhance experimental outcomes.

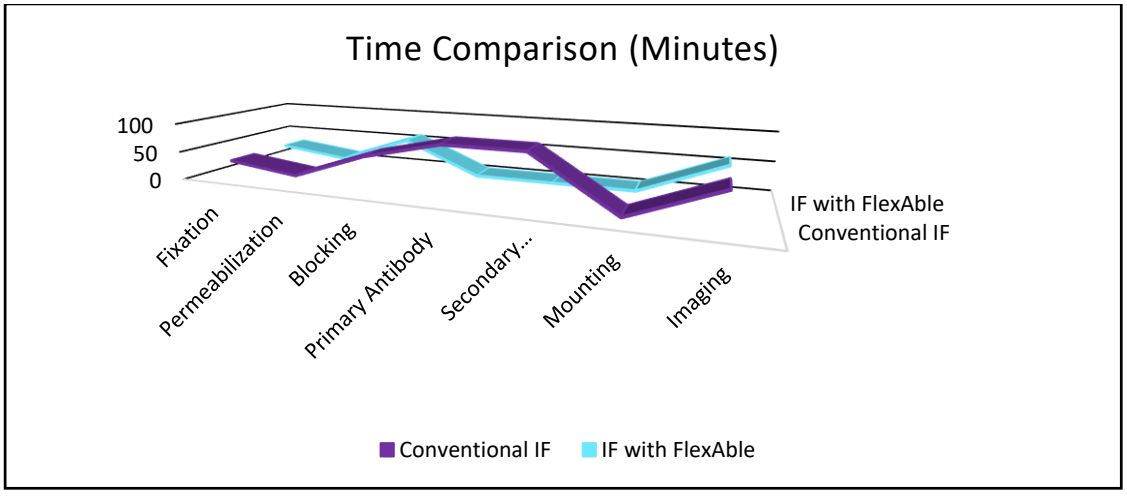

Figure-5. Comparison of the steps of two IF staining studies over time.

Table-2. Comparative Methodological Analysis.

Steps	Conventional IF	IF with FlexAble Kit
Fixation	Cells in six-well plates were fixed with 4% PFA, then rinsed with PBS.	
Permeabilization	Triton X-100 was treated for 15 min. Cells were then rinsed again with PBS.	
Blocking	BSA was incubated for one hour for blocking, then cells were rinsed again with PBS.	
Primer Antibody	Primary antibody was applied to the cells for 1 hour and 30 minutes at room temperature. Then the cells were washed with PBS.	For primary antibody application to the cells for 5 minutes at room temperature, two components from the kit were applied together with the antibody. Then the cells were washed with PBS.
Seconder Antibody	Secondary antibody was applied to the cells for 2 hours at +4ºC and in a dark environment.	A solution from the kit was applied to the cells in a blended form with the secondary antibody for 5 minutes at room temperature in a dark environment.
Mounting	The slides were mounted with mounting solution.	
Imagining	Covered slides were examined using a fluorescence microscope in a dark room.	

Table-3. Comparison of materials.

Steps	List Of Materials	Conventional IF	IF with FlexAble
Fixation	6 Well Plate	+	+
	Eppendorf Tubes	+	+
	Micropipette	+	+
	DU145 Cell Line	+	+
	%4 PFA	+	+
	PBS	+	+
Permeabilization	Triton-X 100	+	+
	PBS	+	+
Blocking	%10 BSA	+	+
	PBS	+	+
Primary Antibody	Caspase-3	+	+
	FlexBuffer	-	+
	FlexLinker	-	+
	PBS	+	+
Secondary Antibody	Alexa Fluor® 488 AffiniPure™ Rabbit Anti-Mouse IgG (H+L)	+	+
	FlexQuencher	-	+
	PBS	+	+
Mounting	DAPI	+	+

RESULTS

The FlexAble Kit used in this study offers a new application compared to IF staining in terms of reducing time, labor and materials compared to the standard IF staining protocol. We compared the data and images obtained from the staining we made using standard IF staining and FlexAble Kit from different perspectives and presented new approaches to the literature.

Efficiency and Yield: Compared to conventional IF staining, it is noteworthy that the ability to perform staining using the 3 components in the FlexAble kit reduces the number of materials and consumables required compared to the standard protocol. This leads to significant cost savings in laboratory management and studies. Secondly, there are also significant differences in antibody binding and antibody binding times between the two staining methods; for example, according to the standard IF staining method, primary and secondary antibody binding is required, and the entire protocol requires approximately 1 day of the scientist responsible for the study (depending on whether the cells are pre-fixed or not, and if the fixation stage is also performed on the same day, the time required for this process will increase even more). In the staining study performed with the FlexAble kit, the process can be completed in approximately 30 minutes to 1 hour with a single kit without spending too much time on primary and secondary antibodies and results suitable for imaging can be obtained (Figure-5).

Functionality Tests: Shows the results obtained by using labeled antibodies in certain analyses, highlighting the differences in performance. As we frequently emphasize in our study, immunofluorescence staining is a laborious application that requires a lot of time and materials and constant monitoring. The FlexAble Kit reduces the use of consumables and special materials such as primary and secondary antibodies for staining, which will be spent on an IF stain, and offers a different perspective and opportunity for scientists and researchers to conduct research effectively in a short time and cost friendly.

User Experience: In terms of usage, there is an advantage between the two protocols not only in terms of the shortening of the time between antibody use and the use of less material for the cells or tissues to be stained, but also in terms of the reduction of process steps in general between the two applications. While the entire process for a scientist performing IF staining in the standard protocol takes about one or one and a half days depending on whether the cells or tissues to be used are fixed or not, staining with the FlexAble Antibody Labeling kit takes between 30 minutes and 1 hour, and effective and high-quality images and data can be obtained in a short time without wasting time and material with applications such as blocking and secondary antibody binding. In addition, when comparing antibody prices between different brands, the fact that 10 labeling can be performed with one kit for the price of only one antibody is another major advantage for researchers (Table-3).

DISCUSSION

The results of our study indicate that the FlexAble Antibody Labeling Kits not only provides an advantage for researchers to be able to use it in multiplex multiplications which in current literature studies, applications performed with the FlexAble Kit are also included in a new way (12-17) and also significantly enhance labeling efficiency and reduce preparation time without compromising the fluorescence intensity and specificity of antibody binding, compared to the conventional direct labeling method with FITC. These findings highlight the challenges of non-specific binding and background fluorescence in traditional methods (18). FlexAble Kits address these issues by providing a streamlined workflow that minimizes such artifacts. Also reported similar improvements in reproducibility and ease of use with kit-based labeling approaches, further reinforcing our observations (19).

The streamlined workflow of FlexAble Kits is one of their most notable advantages. The one-step labeling process eliminates the need for extensive optimization typically required in traditional methods. Eng et al. emphasized the importance of such workflow improvements in enhancing experimental reproducibility (20). Similarly, it is noted that simplifying antibody preparation can significantly reduce the time in complex immunofluorescence experiments (21). FlexAble Kits integrate these improvements by including a purification step that ensures only effectively labeled antibodies are used, maintaining high specificity and reducing background fluorescence.

Another significant advantage observed in our study is the superior stability of fluorescence

signals obtained with FlexAble Kits compared to FITC-labeled antibodies. While FITC signals often degrade over time (22-23). FlexAble Kits maintained stable fluorescence signals over extended periods. This stability is particularly valuable for long-term imaging studies or multi-day experimental workflows. Moreover, Rivest et al. demonstrated that consistent signal stability is critical for high-throughput experiments (24), also a feature of the kit.

Despite these advantages, some limitations were identified. The higher cost per reaction compared to traditional methods presents a challenge, particularly for laboratories with budget constraints. Additionally, the range of fluorescent dyes offered by FlexAble Kits is less extensive than custom labeling methods. This limitation could impact their use in multi-color fluorescence applications requiring specific wavelengths. Expanding the range of dyes, including those compatible with advanced imaging techniques such as super-resolution microscopy, could address this gap. Studies emphasized the need for versatile dye options to meet diverse research requirements (25-27).

Future iterations of FlexAble Kits could focus on optimizing reaction times for even faster workflows and expanding dye compatibility. Broader evaluations across various antibodies, cell types, and experimental conditions would provide deeper insights into their versatility and performance. Dean and Palmer (2014) suggested that innovative labeling technologies could enhance the accuracy of protein localization and molecular interactions (28). Comparative studies have shown that the integration of novel labeling tools can substantially improve data reliability and reproducibility (29 - 30).

CONCLUSION

In conclusion, the FlexAble Antibody Labeling Kits represent a significant advancement in antibody labeling, offering a reliable and efficient alternative to traditional methods. Their ability to streamline workflows, maintain reproducibility, and ensure high-quality results positions them as a valuable tool in modern immunofluorescence research. Future research should validate these findings in broader contexts, including multi-labeling and clinical diagnostic applications, where precision and efficiency are critical. Expanding dye options and reducing costs could further enhance their accessibility and utility across diverse research settings. Furthermore, studies integrating FlexAble Kits into multiplex fluorescence and advanced imaging platforms would provide additional insights into their potential for transforming immunofluorescence methodologies.

Conflict of interest statement : The authors have no conflicts of interest to declare.

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Ethical approval was not required for the writing of the research article.

Consent to Participate and Publish Statement

Consent was required for human subjects in the writing of the research article.

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Availability of Data and Materials

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Author Contributions

KDK: Conceptualization, Writing – review & editing, Investigation, Data curation, Project administration, Supervision, Resources

AT: Formal analysis, Data curation, Resources, Investigation

BH: Conceptualization, Writing – original draft, Investigation, Data curation

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