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A Geno Technology, Inc. (USA) brand name

Protein Folding

Teacher's Guidebook

(Cat. # BE-604)

MATERIALS INCLUDED WITH THE KIT

This kit has enough materials and reagents for 24 students (six groups of four students).

- 1 vial Protein: Albumin
- 1 vial Dye Labeling Agent (FITC)
- 1 vial Dye Labeling Agent ((5/6)-TAMRA-SE)
- 1 vial Dye Solvent
- 1 vial Dye Labeling Buffer
- 6 SpinOUT™ GT-600 Desalting Columns
- 1 vial PAGE: Sample Loading Buffer (2X)
- 30 Centrifuge Tubes (2ml)

SPECIAL HANDLING INSTRUCTIONS

- Store the kit at 4°C in a fridge upon arrival.

ADDITIONAL EQUIPMENT REQUIRED

- 5x5cm pieces of Aluminum Foil
- Protein Electrophoresis Equipment
- Low speed centrifuge for 1.5-2ml tubes
- UV Light Box or UV Lamp

TIME REQUIRED

- **Day 1**: 3 hours

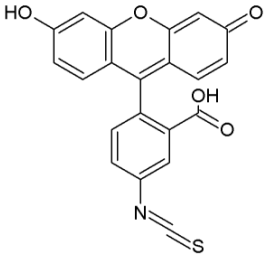
OBJECTIVES

- Label a protein with a fluorescent green or fluorescent red marker.
- Visualize labeled protein using protein electrophoresis.
- Calculate the amount of fluorescent dye bound with a spectrophotometer.
- Understand co-localization by visualizing color change on mixing different labeled proteins.

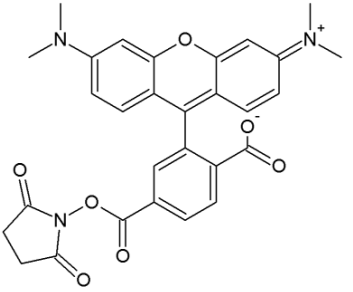
BACKGROUND

Protein labeling is routinely used to label proteins with various markers, including biotin and a multitude of fluorescent markers. The labeling of proteins allows researchers to follow specific molecules using fluorescent microscopy. Normally researchers label specific antibodies and then use these to track proteins of interest.

Fluorescein isothiocyanate (FITC) and *N*-Hydroxysuccinimide (NHS)-ester labeling reagents, such as 5-(and-6)-carboxytetramethylrhodamine succininmidyl ester ((5/6)-TAMRA), are the simplest and most commonly used reagents for labeling proteins. The isothiocyanate group of FITC will crosslink with amino, sulfhydryl, imidazoyl, tyrosyl or carbonyl groups on a protein. However, only the derivatives of primary and secondary amines generally yield stable products. The (5/6)-TAMRA labeling reagent undergoes a cross-linking reaction between the NHS ester on the dye and primary amines on the protein that results in the formation of a stable, covalent amide bond.



Fluorescein Isothiocyanate (FITC)



5-(and-6)-Carboxytetramethylrhodamine succinimidyl ester (5/6)-TAMRA-SE

This kit allows students to label a protein with either the green FITC dye or the red (5/6)-TAMRA. Students will use a desalting column to remove uncoupled dye from the samples and have the option to examine the principle of co-localization using protein electrophoresis.

TEACHER'S PRE EXPERIMENT SET UP

This protocol contains three different procedures for monitoring the labeling of the protein and you can select one, two or all three depending on the time and length of your laboratory sessions.

1. Measure absorbance of the labeled protein versus the original label and protein to determine the degree of labeling.
    a. *This method does not directly show the protein to be labeled.*
    b. *This method does not demonstrate the principle of co-localization.*
2. View the labeled protein under UV light.
    a. *This method does not directly show the protein to be labeled.*
    b. *This method demonstrates the principle of co-localization.*
3. Use protein electrophoresis to view the protein.
    a. *This method shows the protein is directly labeled*
    b. *This method demonstrates the principle of co-localization.*

Acrylamide/Bis-acrylamide is toxic. Always wear gloves and protective clothing when handling the chemicals.

1. Prepare one 10-12% polyacrylamide gel containing 1% SDS (sodium dodecyl sulfate) or use premade electrophoresis gel with 3 sample lanes for each student group. G-Biosciences Protein Electrophoresis Kit is recommended for making your own gel.
2. Prepare the Dye Labeling Buffer. In a 20ml container, add 19ml distilled water and the entire contents of the Dye Labeling Buffer vial. Shake vigorously or vortex until completely dissolved. Store the buffer at 4ºC or on ice. The buffer is stable for 1 week.
3. Add 1.5ml Dye Labeling Buffer from step 2 to the Protein vial. Incubate at room temperature for 5 minutes and then vigorously shake or vortex to dissolve. The concentration of the albumin protein is 2mg/ml. There is an excess of Protein that can be used as a blank.

4. Warm the Dye Labeling agents to room temperature and then briefly centrifuge before use.

5. The Dye Solvent will solidify at 4ºC; warm to room temperature before use.

6. Immediately before use, add 220µl Dye Solvent to the vial of FITC Dye Labeling Agent and pipette up and down until completely dissolved. Store on ice.

7. Immediately before use, add 315µl Dye Solvent to the vial of (5/6)-TAMRA-SE Dye Labeling Agent and pipette up and down until completely dissolved. Store on ice. *The amount of Dye Solvent added to the Fluorescent Dye Labeling Agents ensure that the students will need to add 5µl of each dye to their reactions.*

8. Supply each group with two pieces of 5x5cm (2x2inches) of aluminum foil.

MATERIALS FOR EACH GROUP

Supply each group with the following components. Several components are shared by the whole class and should be kept on a communal table.

- Albumin Protein (2mg/ml) (molecular weight=66340Da)
- Dye Labeling Agent (FITC) (Shared with class)
- Dye Labeling Agent ((5/6)-TAMRA-SE) (Shared with class)
- Dye Solvent (Shared with class)
- Dye Labeling Buffer (Shared with class)
- 1 SpinOUT™ GT-600 Desalting Column
- Sample Loading Buffer (Shared with class)
- 2 5x5cm pieces of aluminum foil
- 3 2ml Centrifuge Tubes

PROCEDURE

This kit is designed for each group of 4 students to label a protein with either a green or red fluorescent dye. Assign either FITC or (5/6)-TAMRA-SE Dye Labeling Agent to each group. Upon the completion of the labeling the different labeled proteins will be shared amongst the groups so that students can compare the green and red and carry out the co-localization experiment.

1. Label a 2ml Centrifuge tube with a group name and the Dye Labeling Agent to be used.

2. Each group transfers 100µl Albumin Protein to a 2ml Centrifuge Tube.

This purified protein has been resuspended directly in Dye Labeling Buffer and is therefore ready to be coupled to a fluorescent dye. In other research scenarios, the

protein may be in a buffer not suitable for protein labeling. If this is the case the protein solution should be dialyzed against a suitable buffer to prepare the sample for coupling.

In this scenario, both fluorescent dyes couple to amine groups, so buffers that contain primary amines, such as Tris or glycine, are not suitable.

3. The next stage is to calculate the optimal amount of fluorescent dye to use. The amount of Dye Labeling Agent to use for each reaction is dependent on the amount of the protein to be labeled. The degree of labeling can be controlled by optimizing the ratio of Dye Labeling Agent to the protein. The guidelines for "optimal molar ratios" are provided in Table 1. They can be varied to alter the degree of labeling.

Dye Labeling Agent	Quantity (mg)	MW	Dye Solvent Volume (µl)	Excitation/ Emission wavelength	Optimal molar ratio to protein
FITC	1	389.5	220	494/ 520nm	20
(5/6)-TAMRA-SE	0.5	527.5	315	546/ 575nm	5 for <100kD protein; 10 for >100kD protein

4. Calculate volume of Fluorescent Dye to add to your protein. Calculate the correct amount for both dyes.:

	For FITC Dye Labeling Agent	For (5/6)-TAMRA-SE Dye Labeling Agent
A. Calculate mmol protein in reaction	**=[protein concentration (mg/ml) x protein volume (ml)]/ MW protein (Da)**	
Write Calculation→	*= [2mg/ml x 0.1ml]/66340* *=3.01x10^{-6}mmol*	*= [2mg/ml x 0.1ml]/66340* *=3.01x10^{-6}mmol*
B. Calculate mmol Dye Labeling Agent required	**= mmol protein x molar ratio**	
Write Calculation→	*= 3.01x10^{-6}mmol x 20* *= 6.02x10^{-5}mmol*	*= 3.01x10^{-6}mmol x 5* *= 1.51x10^{-5}mmol*
C. Calculate µl Dye Labeling Agent required	**=mmol Dye Labeling Agent x MW Dye Labeling Agent x [Dye Solvent Volume (µl) / Dye Labeling Agent Quantity (mg)]**	
Write Calculation→	*= 6.02x10^{-5}mmol x 389.5 x [220µl/ 1mg]* *=5.2µl*	*= 1.51x10^{-5}mmol x 527.5 x [315µl/ 0.5mg]* *=5.3µl*

5. Add the appropriate amount of FITC Dye or (5/6)-TAMRA-SE Labeling Agent to the tube. If necessary round the volume to the nearest microliter (μl).

Store the remaining Albumin Protein, FITC Dye Labeling Agent and (5/6)-TAMRA-SE Dye Labeling Agent in a freezer until required in the "Monitor Protein Labeling using a Spectrophotometer" section.

6. Vortex or briefly shake the tube for 10 seconds to mix the sample.

7. Wrap your centrifuge tube in the supplied aluminum foil to protect the reaction from light and incubate at room temperature for 60 minutes.

The reaction can be incubated overnight at 4ºC (in a fridge) or can be stored in a freezer after the 60 minute incubation until the experiment can be completed.

8. To remove the uncoupled free dye the reaction needs to be passed through a SpinOUT™ GT-600 Desalting Column. Invert the column several times to resuspend the gel material. Spin the column for 10 seconds at 1000xg to allow the gel to collect in the column. Do ***not*** spin too fast or too long.

9. Remove the tip of the column and the cap and let the liquid drain into a 2ml Centrifuge Tube.

10. Buffer Equilibration**:** The SpinOUT™ GT-600 Desalting Column is supplied in deionized water containing a preservative. Equilibrate the column by applying 0.2ml Dye Labeling Buffer to the column. Let the buffer drain into the 2ml Centrifuge Tube. Repeat this process 3 times, and discard the liquid collected in the 2ml Centrifuge Tube.

11. With the column in the 2ml Centrifuge Tube, centrifuge at 1000xg for 2 minute and then discard the liquid collected in the 2ml Centrifuge Tube.

12. Place the column back in the same centrifuge tube. Carefully apply all your sample from step 7 to the center of the column without disturbing the resin bed. Wait for 1-2 minutes.

13. Place the column in a clean 2ml Centrifuge Tube and centrifuge at 1000x g for 4 minutes. Collect the liquid containing the purified labeled protein.

14. Each group requires 30μl FITC labeled protein and 30μl (5/6)-TAMRA-SE labeled protein. Coordinate with another group to get your missing sample.

15. Visualization of Labeled Protein with UV Light: Label three Centrifuge Tubes with "FITC", "TAMRA" and "MIX". Add 10μl FITC labeled protein to the FITC tube, 10μl (5/6)-TAMRA-SE labeled protein to the TAMRA tube and 5μl of each labeled

protein to the MIX tube. Pipette up and down to mix. View the tubes under UV light and record your results in the Results Section.

16. Visualization of Labeled Protein with Protein Electrophoresis: Add 10µl Sample Loading Buffer to each tube from step 15, pipette up and down to mix. Place all three tubes in a boiling waterbath for 3-5 minutes. Briefly centrifuge to bring down the condensation

17. Load 10µl of each sample onto the prepared protein gels according to your supervisor's instructions. Load the "FITC", "TAMRA" and then "MIX" samples. Run the gel according to your supervisor's instructions until the blue dye front is ⅔ of the length of the gel.

The labeled proteins will be visible during the migration through the gel. The gel does not have to be run the full length. If time is short, stop the electrophoresis once the labeled proteins have migrated >1cm into the separating gel.

18. Remove the gel from the apparatus and place on a UV light box. Record your results in the results section.

19. Monitor Protein Labeling using a Spectrophotometer: Label two Centrifuge Tubes with "Unlabeled Protein" and "Labeled Protein".

20. Add 50µl unlabeled original Albumin Protein to the "Unlabeled Protein" tube and 50µl of your labeled protein to the "Labeled Protein" tube.

21. Add 450µl distilled water to the "Unlabeled Protein" and "Labeled Protein" tubes.

NOTE: *For optimal results, use a microplate reader and add only 150µl distilled water to your 50µl samples. If 500µl is too small a volume to be measured in your spectrophotometer then dilute the sample with 950µl. This may generate very low readings.*

22. Measure the absorbance of the FITC labeled protein at 494nm. And the TAMRA labeled protein at 546nm. Zero the spectrophotometer with the "Unlabeled Protein" tube. Record the values in the results section.

23. Calculate the Degree of Protein Labeling by following the instructions in the results section.

RESULTS, ANALYSIS & ASSESSMENT

Appearance under UV light:

"FITC":_____ *(Bright Green)*

"TAMRA": _____ *(Red)*

"MIX": _____ *(Yellowy Green))*

Does this result demonstrate that the protein is labeled with the appropriate dye?

The result strongly suggests that the protein is labeled with the appropriate dye, however this is assuming that all the free, uncoupled dye was removed by the Desalting column. The color could be a result of the presence of free dye.

Explain the color of the "MIX" sample and its importance with respect to co-localization of proteins:

The "MIX" sample is yellow due to the combination of the red and green dyes. This means that the if two different dye labeled proteins interact in a cell then when viewed through a fluorescent microscope their site of interaction will glow yellow as opposed to green or red.

Describe the result of the protein electrophoresis below and explain if this result shows that the protein is labeled with the dye:

There were three bands on the gel of the same molecular weight. The FITC band glowed green, the TAMRA band red and the MIX band yellow/green. Protein electrophoresis separates proteins based on their size. Any unbound dye would rapidly pass through the gel, away from the slower migrating labeled protein. This means that the fluorescent band seen is the protein and is labeled with the dye.

Calculate the molar ratio of dye:protein.

Measure the absorbance of the labeled protein at the fluorescent dyes excitation maximum (A_{max}). For FITC this is 494nm and TAMRA this is 546nm.

Absorbance of FITC labeled protein at 494nm:_____

Absorbance of TAMRA labeled protein at 546nm:_____

Calculate the molarity of the protein:

Molarity of protein (M) = $\frac{\text{moles of solute}}{\text{liters of solution}}$

= $\frac{\text{From page 4 converted to moles}}{100\mu\text{l reaction mix in liters}}$

= $\frac{3.01 \times 10^{-9}}{0.0001}$

= 3.01×10^{-5}

Calculate the degree of labeling:

Moles dye per mole protein = $\frac{A_{max} \text{ of labeled protein} \times \text{dilution factor}}{\varepsilon' \times \text{protein concentration (M)}}$

ε'= molar extinction coefficient of the fluorescent dye

For FITC ε'= 68,000$M^{-1}cm^{-1}$; for TAMRA ε'= 95,000$M^{-1}cm^{-1}$

Dilution factor is the factor used in step 21.

Moles dye per mole protein = $\frac{0.836}{(68{,}000 \times 3.01 \times 10^{-5})} \times 10$

=4.08moles of FITC per mole of protein.

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- Label a protein with a fluorescent green or fluorescent red marker.
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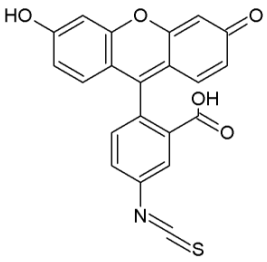
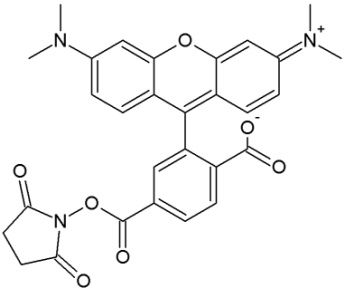


Fluorescein Isothiocyanate (FITC)



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- Dye Solvent (Shared with class)
- Dye Labeling Buffer (Shared with class)
- 1 SpinOUT™ GT-600 Desalting Column
- Sample Loading Buffer (Shared with class)
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This purified protein has been resuspended directly in Dye Labeling Buffer and is therefore ready to be coupled to a fluorescent dye. In other research scenarios, the protein may be in a buffer not suitable for protein labeling. If this is the case the protein solution should be dialyzed against a suitable buffer to prepare the sample for coupling.

In this scenario, both fluorescent dyes couple to amine groups, so buffers that contain primary amines, such as Tris or glycine, are not suitable.

3. The next stage is to calculate the optimal amount of fluorescent dye to use. The amount of Dye Labeling Agent to use for each reaction is dependent on the amount of the protein to be labeled. The degree of labeling can be controlled by optimizing the ratio of Dye Labeling Agent to the protein. The guidelines for “optimal molar ratios” are provided in Table 1. They can be varied to alter the degree of labeling.

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4. Calculate volume of Fluorescent Dye to add to your protein. Calculate the correct amount for both dyes.:

	For FITC Dye Labeling Agent	For (5/6)-TAMRA-SE Dye Labeling Agent
A. Calculate mmol protein in reaction	**=[protein concentration (mg/ml) x protein volume (ml)]/ MW protein (Da)**	
Write Calculation→		
B. Calculate mmol Dye Labeling Agent required	**= mmol protein x molar ratio**	
Write Calculation→		
C. Calculate µl Dye Labeling Agent required	**=mmol Dye Labeling Agent x MW Dye Labeling Agent x [Dye Solvent Volume (µl) / Dye Labeling Agent Quantity (mg)]**	
Write Calculation→		

5. Add the appropriate amount of FITC Dye or (5/6)-TAMRA-SE Labeling Agent to the tube. If necessary round the volume to the nearest microliter (µl).

Store the remaining Albumin Protein, FITC Dye Labeling Agent and (5/6)-TAMRA-SE Dye Labeling Agent in a freezer until required in the "Monitor Protein Labeling using a Spectrophotometer" section.

6. Vortex or briefly shake the tube for 10 seconds to mix the sample.

7. Wrap your centrifuge tube in the supplied aluminum foil to protect the reaction from light and incubate at room temperature for 60 minutes.

The reaction can be incubated overnight at 4ºC (in a fridge) or can be stored in a freezer after the 60 minute incubation until the experiment can be completed.

8. To remove the uncoupled free dye the reaction needs to be passed through a SpinOUT™ GT-600 Desalting Column. Invert the column several times to resuspend the gel material. Spin the column for 10 seconds at 1000xg to allow the gel to collect in the column. Do ***not*** spin too fast or too long.

9. Remove the tip of the column and the cap and let the liquid drain into a 2ml Centrifuge Tube.

10. Buffer Equilibration**:** The SpinOUT™ GT-600 Desalting Column is supplied in deionized water containing a preservative. Equilibrate the column by applying 0.2ml Dye Labeling Buffer to the column. Let the buffer drain into the 2ml Centrifuge Tube. Repeat this process 3 times, and discard the liquid collected in the 2ml Centrifuge Tube.

11. With the column in the 2ml Centrifuge Tube, centrifuge at 1000xg for 2 minute and then discard the liquid collected in the 2ml Centrifuge Tube.

12. Place the column back in the same centrifuge tube. Carefully apply all your sample from step 7 to the center of the column without disturbing the resin bed. Wait for 1-2 minutes.

13. Place the column in a clean 2ml Centrifuge Tube and centrifuge at 1000x g for 4 minutes. Collect the liquid containing the purified labeled protein.

14. Each group requires 30µl FITC labeled protein and 30µl (5/6)-TAMRA-SE labeled protein. Coordinate with another group to get your missing sample.

15. Visualization of Labeled Protein with UV Light: Label three Centrifuge Tubes with "FITC", "TAMRA" and "MIX". Add 10µl FITC labeled protein to the FITC tube, 10µl (5/6)-TAMRA-SE labeled protein to the TAMRA tube and 5µl of each labeled protein to the MIX tube. Pipette up and down to mix. View the tubes under UV light and record your results in the Results Section.

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17. Load 10µl of each sample onto the prepared protein gels according to your supervisor's instructions. Load the "FITC", "TAMRA" and then "MIX" samples. Run the gel according to your supervisor's instructions until the blue dye front is ⅔ of the length of the gel.

The labeled proteins will be visible during the migration through the gel. The gel does not have to be run the full length. If time is short, stop the electrophoresis once the labeled proteins have migrated >1cm into the separating gel.

18. Remove the gel from the apparatus and place on a UV light box. Record your results in the results section.

19. Monitor Protein Labeling using a Spectrophotometer: Label two Centrifuge Tubes with "Unlabeled Protein" and "Labeled Protein".

20. Add 50µl unlabeled original Albumin Protein to the "Unlabeled Protein" tube and 50µl of your labeled protein to the "Labeled Protein" tube.

21. Add 450µl distilled water to the "Unlabeled Protein" and "Labeled Protein" tubes.

NOTE: *For optimal results, use a microplate reader and add only 150µl distilled water to your 50µl samples. If 500µl is too small a volume to be measured in your spectrophotometer then dilute the sample with 950µl. This may generate very low readings.*

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23. Calculate the Degree of Protein Labeling by following the instructions in the results section.

RESULTS, ANALYSIS & ASSESSMENT

Appearance under UV light:

"FITC":_____ *(Bright Green)*

"TAMRA": _____ *(Red)*

"MIX": _____ *(Yellowy Green))*

Does this result demonstrate that the protein is labeled with the appropriate dye?

_____

_____

_____

_____

_____

Explain the color of the "MIX" sample and its importance with respect to co-localization of proteins:

_____

_____

_____

_____

_____

Describe the result of the protein electrophoresis below and explain if this result shows that the protein is labeled with the dye:

_____

_____

_____

_____

_____

Calculate the molar ratio of dye:protein.

Measure the absorbance of the labeled protein at the fluorescent dyes excitation maximum (A_{max}). For FITC this is 494nm and TAMRA this is 546nm.

Absorbance of FITC labeled protein at 494nm:_____

Absorbance of TAMRA labeled protein at 546nm:_____

Calculate the molarity of the protein:

Molarity of protein (M) = moles of solute / liters of solution

= From page 4 converted to moles / 100µl reaction mix in liters

_____

_____

_____

_____

_____

Calculate the degree of labeling:

Moles dye per mole protein = A_{max} of labeled protein x dilution factor / ε' x protein concentration (M)

ε'= molar extinction coefficient of the fluorescent dye

For FITC ε'= 68,000$M^{-1}cm^{-1}$; for TAMRA ε'= 95,000$M^{-1}cm^{-1}$

Dilution factor is the factor used in step 21.

Moles dye per mole protein =

_____

_____

_____

_____

_____

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