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Cell DIVE™ Platform | Ab Conjugation: Initial Conjugation & Scale up Conjugation

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1 Works for me dx.doi.org/10.17504/protocols.io.bp55mq86

Human BioMolecular Atlas Program (HuBMAP) Method Development Community GE Research

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ABSTRACT

Scope

The purpose of this protocol is to conjugate purified antibodies to Cy dyes. Antibody conjugation involves combining dyes with antibodies to achieve a fluorescently labeled antibody. Each antibody will be labeled with a molar equivalent of dye; the quantitative output of this is deemed D/P. In the initial conjugation step, each antibody will be labeled with differing D/P levels and validated to determine which level is optimal.

ATTACHMENTS

Cell_DIVE-manual-Ab-conjugation-chemistry_final_version.pdf

DOI

dx.doi.org/10.17504/protocols.io.bp55mq86

PROTOCOL CITATION

Anup Sood, Eric Williams, Liz McDonough 2021. Cell DIVE™ Platform | Ab Conjugation: Initial Conjugation & Scale up Conjugation. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bp55mq86

KEYWORDS

Antibody, Antibodies, conjugation, Cell Dive, Cy dye

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OWNERSHIP HISTORY

Nov 27, 2020 Julia Rossmanith protocols.io

Dec 11, 2020 Liz McDonough GE Research

PROTOCOL INTEGER ID

44957

GUIDELINES

Associated Documents

A	B
Document File Name	**Document Title**
Form-1	Cy2/Cy3/Cy5/Cy7 Amount of Dye to Add
Form-2	Cy3 Direct Conjugate Dye to Protein Calculation
Form-3	Cy5 Direct Conjugate Dye to Protein Calculation
Form-4	Amount of Antibody Required For Initial Direct Conjugation
Form-5	Cy2 Direct Conjugate Dye to Protein Calculation
Form-6	Cy7 Direct Conjugate Dye to Protein Calculation
Form-7	Amount of dye to add – fast form

Definitions & Descriptions

Definitions

A	B
Definition/Acronym	**Definitions**
BSA	Bovine Serum Albumin
PBS	Phosphate Buffered Saline
D/P	Dye to Protein Ratio
Efficiency	A percentage value as determined by the amount of dye labeled to the antibody compared to the amount that was added to it
dH_2O	Distilled water
DMSO-d6	Deuterated Dimethyl Sulfoxide
$K_2Cr_2O_7$	Potassium dichromate – Calibration Fluid for the NanoDrop
DC	Direct Conjugate
nm	Nanometer
A_{280}	Protein absorbance at 280 nm
A_{Dye}	Dye absorbance

Descriptions

In a given assay, when an antibody gets down selected from P/S validation proceed to Section 8 for initial conjugation. This section describes the process of conjugating an antibody at two D/P levels.

After the conjugate is made, a biologist will validate the two different D/P and select the best D/P based on specificity and sensitivity. After the D/P down-selection, the user proceeds to Section 9 for the process of scaling up the conjugate (if needed).

If antibody has been conjugated previously and it is in one of our DC catalogues, please proceed immediately to Section 9 for the scale up conjugation protocol; you do not need to do the pilot conjugation for validated DCs.

Troubleshooting

Citation: Anup Sood, Eric Williams, Liz McDonough (01/27/2021). Cell DIVEÃçÂÂç Platform | Ab Conjugation:ÃÂ Initial Conjugation & Scale up Conjugation. https://dx.doi.org/10.17504/protocols.io.bp55mq86

A	B	C
Problem	**Cause**	**Next Steps**
If conjugation efficiency is <10%	There could be glycerol	Call vendor and confirm. If yes proceed to purify as written in section 4.9 of Antibody purification SOP. Re-conjugate the same conjugate to a desired D/P.
If conjugation efficiency is <10%	Antibody in Tris buffer – look at data sheet to confirm	Proceed to purify/buffer exchange as written in section 4.9 of Antibody purification manual. Re-conjugate the same conjugate to a desired D/P
If conjugation efficiency is >10% <40%	Dye calculation errors	Check spreadsheet for errors, if needed re-measure dye concentration
If purification yield is < 50%	Precipitation	Do conjugation at 500 µg/mL
If conjugate precipitates after purification	Concentration might be too high	Dilute the conjugate to 100 µg/mL and when conjugate is made next time – make at 500 µg/mL and read the conjugates after purification immediately and dilute them to 100 µg/mL
D/P ratios are out of spec (ie, the D/P ratio exceeds the permissible std dev)	Dye concentration read out is not correct	Re-measure dye concentration and repeat

MATERIALS TEXT

Components

Required Materials

A	B
Material	**Intended Use**
Pipettes (2-1000µL)	Deliver liquid volumes (µL) for antibody conjugation
Pipette Tips (0.5-1000 µL)	Used for pipettes
Nitrile Gloves	Personal Protective Equipment
Plastic Bottle	Used to mix solutions
Timer	Used to time incubations
15 mL conicals	Collection of purified conjugates
Eppendorf tubes	Used to store conjugation reactions
Amber Eppendorf tubes	Used for long term storage of conjugated antibodies
Small Kimwipes	Used to clean NanoDrop and pipette tips

Required Reagents

Note: Follow proper disposal for all reagents. Safety information and disposal is located in the laboratory.

A	B
Reagent/Media/Controls/Probes	**Definitions**
Zeba Desalt Spin Columns, 0.5 mL (Pierce/Fisher, 89883)	• Quantity: 1 column = 0.5 mL • Storage: 4°C • Composition: Resin slurry contains 0.05% Sodium Azide as a preservative. • Expiration Date: Do not use after date stamped on manufacturer's label

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20X Borate Buffer (Fisher Scientific, PI28341	• Quantity: 500 mL • Composition: 1M borate • Storage: Room Temperature • Expiration date: one year from date of receipt
10X PBS (Sigma, P7059)	• Quantity: 1 L • Storage: Room Temperature (liquid) • Composition: 10X concentrate phosphate buffered saline • Expiration date: one year from date of receipt
Cy2- Bis NHS Ester Dye (GEHC, PA12000)	• Quantity: 5.0 mg • Storage: 4°C • Composition: Cy2 Bisfunctional dye • Expiration Date: Do not use after date stamped on manufacturer's label
Cy3- Bis NHS Ester Dye (GEHC, PA13000)	• Quantity: 5.0 mg • Storage: 4°C • Composition: Cy3 Bisfunctional dye • Expiration Date: Do not use after date stamped on manufacturer's label
Cy5-Bis NHS Ester Dye (GEHC, PA15000)	• Quantity: 5.0 mg • Storage: 4°C • Composition: Cy5 Bisfunctional dye • Expiration Date: Do not use after date stamped on manufacturer's label
Cy7-Bis NHS Ester Dye (GEHC, PA17000)	• Quantity: 5.0 mg • Storage: 4°C • Composition: Cy7 Bisfunctional dye • Expiration Date: Do not use after date stamped on manufacturer's label
Dimethyl Sulfoxide- d_6, 100% D (Sigma, 453323)	• Quantity: 0.25 mL, 10 per pkg. • Storage: Room Temperature • Composition: di[(2H)Methyl] Sulfoxide • Expiration Date: Do not use after date stamped on manufacturer's label
Bovine Serum Albumin (Sigma, Catalog#: A2153)	• Quantity: 50 g • Storage: 2-8°C, light sensitive • Composition: ≥96% lyophilized powder • Expiration date: Retest date five years from manufacturers QC date
Sodium Azide 0.1 M (Sigma, 08591)	• Quantity: 1 mL • Storage: 4°C • Composition: Sodium Azide Solution (powder) • Expiration Date: Do not use after date stamped on manufacturer's label
PD-10 Desalting Columns (GE Healthcare Cat# 17-0851-01)	• Pack of 30 columns • Storage: Room Temperature • Composition: Sephadex G-25 in column storage solution • Expiration Date: Do not use after date stamped on manufacturer's label

If a NanoDrop is being used, the following kits are also required –

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A	B
Reagent/Media/Controls/Probes	**Definitions**
CF-1 (K2Cr2O7) NanoDrop 2000C Calibration Kit (ThermoFisher, CF-1)	• Quantity: 6 vials per pkg, 1 uL each • Storage: Room Temperature • Composition: Potassium dichromate .07-.10%, Perchloric acid .02-.04%, water 99.8% • Expiration Date: Do not use after date stamped on manufacturer's label
PR-1 Pedestal Reconditioning Kit (ThermoFisher, PR-1)	• Quantity:25 test swaps and 10ml of paste • Storage: Room Temperature • Composition: 10-30% Solvent Naphtha, 5-10% Petroleum distillates (PR-1 reconditioning compound) • Expiration Date: Do not use after date stamped on manufacturer's label

Required Equipment

A	B
Equipment	**Intended Use**
NanoDrop 2000c	Used for measuring Absorbance of Proteins (280nm) and Dye's (variable wavelength)
Microcentrifuge	Used for spinning antibodies down prior to conjugation and for pilot conjugation purification
Fisher Vortex Genie 2	Used for mixing of solutions
Denver Instruments UB-10	pH meter used to check pH of reagents
Analytical Balance	Used to weigh reagents
Fixed Speed Centrifuge	Used to spin down solutions after mixing via vortex
Laptop connected to NanoDrop	Drives NanoDrop software

Reagent Prep

Note: Follow proper disposal for all reagents. Safety information and disposal is located in the laboratory. Be sure to label all reagents with appropriate name, date made, expiration date and safety label.

A	B
Reagents	**Definitions**

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1X PBS	• Quantity: 1 L • Composition: 100 mL 10X PBS, 900 mL dH2O • Storage: Room Temperature. • Expiration Date: One year from date of stock receipt. • Mix thoroughly into a 1 L bottle.
Cy Dye 1X Stock Solution	• Take a pinch from the 5 mg vial and dissolve in 40 µL DMSO • Storage Conditions: 4°C, wrap vial in parafilm and store in aluminum storage sleeve • Expiration: One week after creation • Thaw by placing aluminum sleeve containing the vial on the bench top for 10 minutes at 21-27°C. Vortex and spin before using
Dye Concentration Measurement Solution	• Quantity: 500 µL • Composition: 2 µL Cy Dye 1X Stock Solution, 498 µL 1X PBS • Storage Conditions: Store at 21-27°C • Expiration date: one hour from time and date made • Prepare solution immediately before use, discard after one hour
Cy Dye 0.1X Working Solution	• Quantity: Volumes and Working Dilutions will be dependent on amount of antibody being conjugated • Composition: 1 Part Cy Dye 1X Stock Solution, 9 Parts 1X PBS • Storage conditions: Discard immediately after use • Expiration: Discard immediately after use
Cy Dye 0.2X Working Solution	• Quantity: Volumes and Working Dilutions will be dependent on amount of antibody being conjugated • Composition: 1 Part Cy Dye 1X Stock Solution, 4 Parts 1X PBS • Storage conditions: Discard immediately after use • Expiration: Discard immediately after use
Cy Dye 0.33X Working Solution	• Quantity: Volumes and Working Dilutions will be dependent on amount of antibody being conjugated • Composition: 1 Part Cy Dye 1X Stock Solution, 2 Parts 1X PBS • Storage conditions: Discard immediately after use • Expiration: Discard immediately after use

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Cy Dye 0.5X Working Solution	• Quantity: Volumes and Working Dilutions will be dependent on amount of antibody being conjugated • Composition: 1 Part Cy Dye 1X Stock Solution, 1 Part 1X PBS • Storage conditions: Discard immediately after use • Expiration: Discard immediately after use
Stabilizing Solution (1% BSA/0.45% Sodium Azide)	• Quantity: 1000 µL • Composition: 10 mg BSA, 693 uL 0.1M Sodium Azide; Dilute up to 1000 µL with 1X PBS • Storage Conditions: 2-8°C • Expiration: Two months from date of creation

SAFETY WARNINGS

Safety warnings and precautions

Warning: For research use only.

Cell DIVE software and workflows are for internal research use only and not for third party service use or clinical diagnosis. Do not use internally or externally in humans or animals. All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory coats, safety glasses, and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water.

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

BEFORE STARTING

Critical Parameters

Please take particular note of the following instructions regarding critical steps:

- It is essential to read the complete instruction booklet before starting work.
- These instructions have only been validated on formalin-fixed paraffin embedded tissue sections.
- Unless noted, it is essential to allow reagents to reach room temperature prior to use.
- Mix samples and all reagents thoroughly before use.
- Avoid extensive exposure of fluorescent reagents to ambient light.

1 Please select between the following cases:
Step 1 includes a Step case.
Pilot Conjugation
Scale-Up Conjugation

Determining molar concentration of 1X Cy Dye via NanoDrop 2000c

step case

Pilot Conjugation

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2

Note: In case if your institution does not have NanoDrop, please use a UV spectrophotometer of your choice.

For Cy2 - measure absorption at 489 nm. The Cy2 extinction coefficient is 150,000. Divide absorption at 550 nm by 0.15 to get concentration in µM.

Molar Concentration (mM) = (Average µM concentration X 250 dilution factor)/1000.

For Cy3 - measure absorption at 550 nm. The Cy3 extinction coefficient is 150,000. Divide absorption at 550nm by 0.15 to get concentration in µM.

Molar Concentration (mM) = (Average µM concentration X 250 dilution factor)/1000.

For Cy5 - measure absorption at 650 nm. The Cy5 extinction coefficient is 250,000. Divide absorption at 650 nm by 0.25 to get concentration in µM.

Molar Concentration (mM) = (Average µM concentration X 250 dilution factor)/1000.

For Cy7 - measure absorption at 750 nm. The Cy7 extinction coefficient is 200,000. Divide absorption at 650 nm by 0.2 to get concentration in µM.

Molar Concentration (mM) = (Average µM concentration X 250 dilution factor)/1000.

3 Open the NanoDrop 2000c software version 7.2.1.1 program located on the desktop of the NanonDrop computer.

4 From the home screen select “Proteins and Labels”.

5 Select IgG under “Type”, as per Figure 1.1.

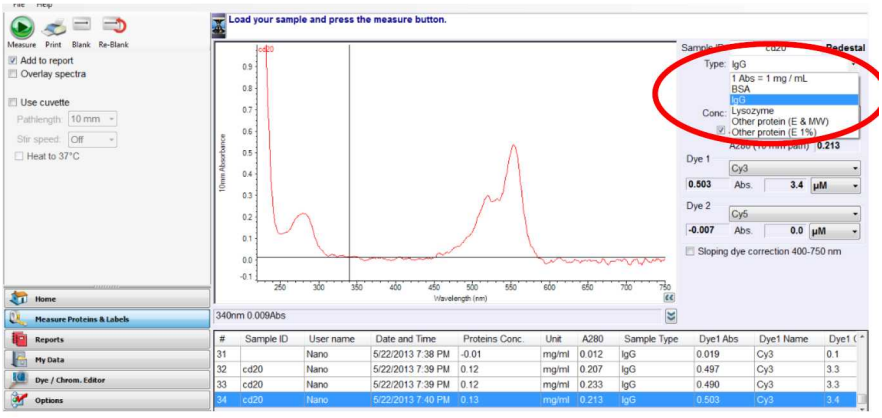

Figure 1.1 Selecting IgG as the protein Type

6 Select the dye to be measured from the dropdown menu for “Dye1,” as per (1) on Figure 1.2.

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NOTE 1: Cy2 is not a standard option in the NanoDrop software. You will need to add Cy2 to the dye list. Consult the instrument software manual for specific instructions.

NOTE2: Cy7 is incompatible with the “Proteins and Labels” Program. Use the “UV-Vis” program to read Cy7 dye and conjugates.

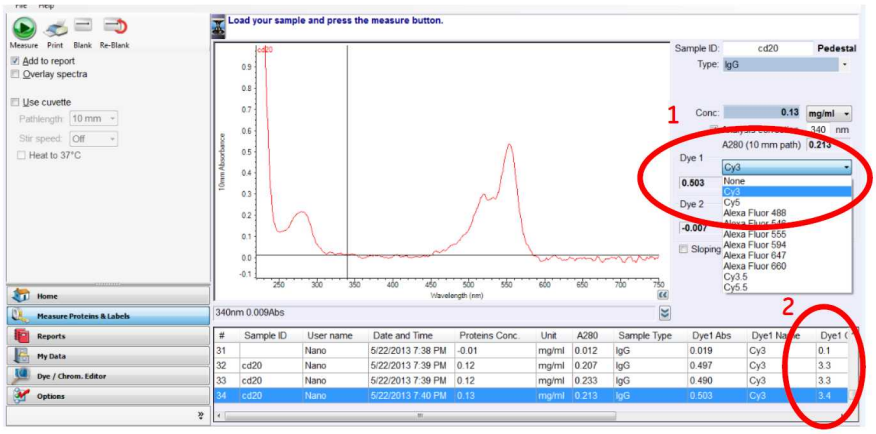

Figure 1.2: Setting Dye Parameters on NanoDrop

7 Measure **2 µl fresh 1X PBS** and place on NanoDrop pedestal to blank the instrument.

8 Select “Blank.”

9 After the measurement completes wipe the pedestal with a Kimwipe.

10 Measure **2 µl fresh 1X PBS** and place on NanoDrop pedestal to measure the baseline.

11 Select “Measure.”

12 After the measurement completes wipe the pedestal with a Kimwipe.

13 Refer to Figure 1.3: Ensuring a Stable Baseline on the NanoDrop.

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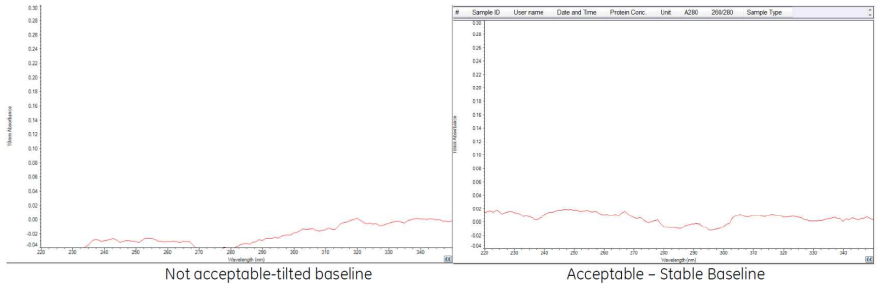

Figure 1.3: Ensuring a Stable Baseline on the NanoDrop

- A Stable Baseline should read an absorbance between 0.02 and –0.02, with no tilt in the horizontal line.
- An Unstable Baseline will look tilted or veer outside of the acceptable -0.02 and +0.02 acceptable range.

14 Measure **2 µl of the dye concentration measurement solution** (Section 'Materials' for preparation, in µM) 5 times, taking 5 independent readings.

- Spoiler - the 'dye concentration measurement solution' is 2µL of the Cy dye stock solution mixed with 498µL of PBS.

15 The instrument will calculate the dye concentration.

16 Refer to (2) of Figure 1.4: Setting Dye Parameters on NanoDrop.

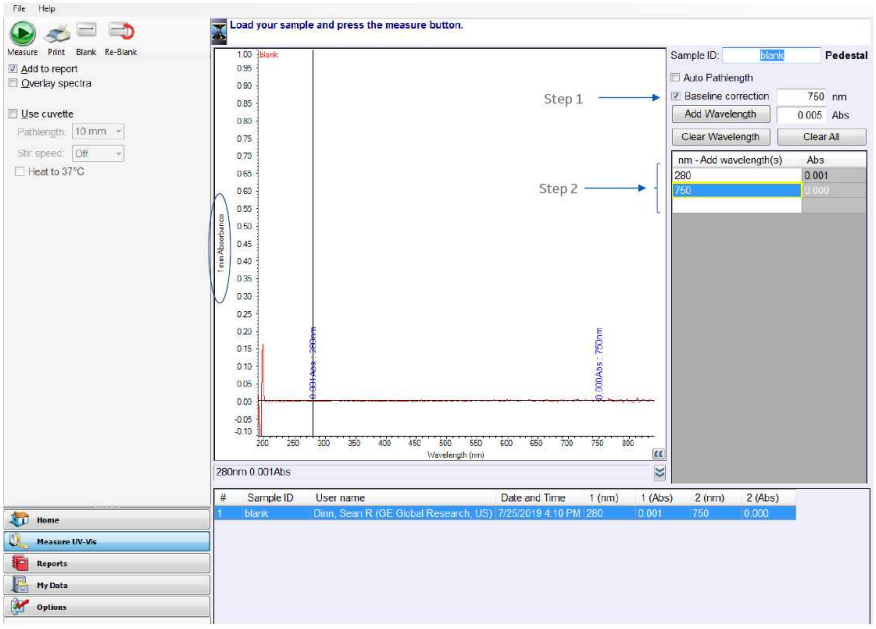

Figure 1.4: Measuring Cy7 on the NanoDrop

17 Cy7 measurements: The measurements for Cy7 dye and conjugates are slightly different.

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17.1 From the home screen select “UV-Vis”.

17.2 Change the “Baseline correction” wavelength to a wavelength where the signal is expected to be 0. Refer to Figure 1.4, Step 1.

17.3 Add 280 and 750 nm to the list of wavelengths to be measured. Refer to Figure 1.4, Step 2.

17.4 In the UV-Vis program, the absorbance is displayed for a 1 mm path length and the dye concentration is not automatically calculated. The dye concentration for Cy7 is calculated as follows: Dye concentration (µM) = $Absorbance_{750nm}$*50

18 To calculate the molar concentration of the Cy dye 1X stock solution:

Molar Concentration (mM) = (Average µM concentration X 250 dilution factor)/1000

19 Write the calculated concentration on the aluminum storage sleeve along with the date.
This Molar Concentration (mM) will be used as the Dye Conc. in Form-1 and Form-7.

- Note - preferred but not necessary; Cy dye 1x stock solution concentration is preferred to be ~3 mM. If the concentration is over 3.5 mM, dilute with DMSO, if the concentration is below 2 mM, add more dye powder and return to step 2.

Preparing calculations for pilot conjugations

20 Refer to Table 2.1, for specific parameters for the antibody to be conjugated.

Dye	# of D/P Ratios to test	Target Initial D/P	Target Initial Efficiency	Acceptable D/P Range
Cy2	2	3.5/5.0	75%	3.0-6.0
Cy3	2	3.5/5.0	75%	3.0-6.5
Cy5	2	1.5/3.0	75%	1.5-4.5
Cy7	2	1.5/3.0	75%	1.5-4.5

Table 2.1: Parameters to use for direct conjugation

NOTE: In the event antibody purification yield is low or the vendor supplies insufficient quantity to perform 2 D/P per dye, inform the study coordinator and, if approved, continue conjugating 1 D/P per dye.

21 The pilot conjugations will be used to determine the optimal D/P for the specific antibody tested. Every antibody will have an optimal D/P.

22 Determine the minimum quantity to conjugate. Be sure to save at least **5 µg unconjugated primary antibody** to be validated alongside the direct conjugate.

- Optimally, **45 µg** reactions should be performed; however, a minimum of **30 µg** is required for every conjugation, regardless of study.
- If the conjugation will be used beyond initial testing, i.e. for a multiplexing run, refer to Form-4: Amount of Antibody

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Required for Initial Direct Conjugation, to input the data and calculate the optimal amount of antibody to conjugate. Consult the study coordinator to determine the number of slides required for the study. (This form will calculate the minimum amount of antibody needed to conjugate based on the study. If purification is required, refer to the purification manual.)

23 Use Form-7 to calculate the volume of antibody, 20X borate, 1X PBS and dye needed for the pilot reaction

- Input any cell that is colored blue
- "Dye purity" is the percent (%) reactive dye information that will be on the Certificate of Analysis and is lot dependent. Enter the information on Form 7 in decimal form (i.e. 90.2% = 0.902). If the information is not available, use the following website to look up the CoA using the lot#: https://www.cytivalifesciences.com/en/us/support/quality/certificates
- "Loading" is the initial ratio of dye to antibody that is used in the conjugation. The loading is calculated as: Initial D/P divided by expected efficiency (expressed as a decimal). Refer to Table 2.1 for the appropriate values. For example, if targeting a Cy3 labeling at a D/P of 3.5, the "Loading" would be 4.67 (3.5/0.75).
- Choose a dye dilution that will allow you to add a minimum of 2 µL of solution while not overly diluting the entire sample. As you adjust the "dye dilution" cell on Form-7, the "µL dye" will change accordingly. This volume is the amount of the diluted dye that you need to add. See Section 'Materials' for the preparation of the working dye solutions. If the dilution needed is not described in Section 'Materials', adjust the calculations accordingly. You should prepare at least twice the volume of working solution that will need to be added to the conjugation.
- If there is enough antibody, repeat the calculations for the second D/P target.
- Refer to Figure 2.1, for an example of calculating these values.
- On Form-7, there are two choices of calculations depending on your starting antibody concentration.

Enter information into blue boxes

Ab	Conc (mg/mL)	ug	uL	Dye	Conc (mM)	Dye purity	Loading	uL dye	dye dilution	PBS (µL)	Borate (20x) µL	Final volume µL	Final Ab conc (mg/mL)
Ab1	0.95	106	111.5789	Cy5	1.76	0.902	1.9	1.636962	0.5	0.00	5.96	119.17	0.889450968
Ab2	1.6	226	141.25	Cy5	1.76	0.902	4.5	4.133044	1	69.32	11.30	226.00	1

Figure 2.1: Antibody Conjugation Preparation

Antibody Pilot Conjugation 30m 16s

24 Remove the antibody from it's storage location and allow it to equilibrate to **Room temperature** for **00:45:00** 45m

25 Remove the aluminum jacket containing the Cy Dye 1X Stock Solution from its storage location.

If the dye is being removed from the refrigerator allow it to thaw at **Room temperature** for approximately **00:15:00** , while remaining in the aluminum jacket.

26

Combine volumes specified in **go to step #23** for the antibody, fresh 1X PBS and borate into a 1.5 mL eppendorf tube using a pipette.

27 Vortex the mixture for **00:00:03** at a moderate speed to thoroughly mix. 3s

28 5s

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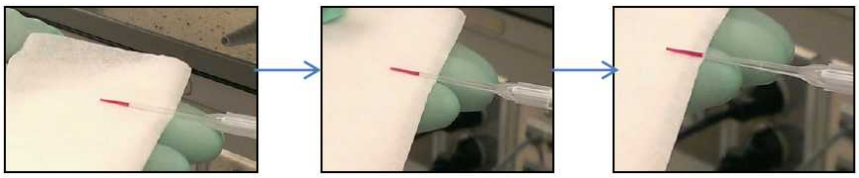
Spin the mixture in the Fixed Speed Centrifuge for **00:00:05** .

Do not allow the mixture to incubate for **more than 5 minutes before adding** the dye working dilution, as the antibody structure could be compromised.

29 Make the dye dilution calculated in **go to step #23** carefully by adding the calculated amount of Cy Dye 1X Stock Solution to 1X PBS.

To ensure that unwanted dye has not stuck to the outside of the pipette tip, gently wipe the outside of the pipette tip with a Kimwipe before diluting in 1X PBS. See image below:



30 Vortex the dye working solution for **00:00:03** at a speed setting of 9.

3s

31

5s

Spin the solution in the fixed speed centrifuge for **00:00:05** .

32

Add the appropriate amount of working Dye Solution, calculated in **go to step #23** , **within 1 minute** to the antibody/borate mixture to prevent dye hydrolysis.

33 Place solution in an antibody cardboard box in a drawer containing no light.

34

30m

Incubate at **Room temperature** for **00:30:00** .

Use a timer to ensure reaction does **not go over 30 minutes**.

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Preparing Zeba Spin Columns for Direct Conjugate Purification

35 Begin this process immediately after conjugate incubation has commenced (step 33).

36 Remove two Zeba Spin 0.5 mL columns from their packaging.

If doing multiple conjugations simultaneously, prepare 2 columns per conjugation.

37 Remove and dispose of bottom closures and loosen caps.

38 Place columns into 1.5 mL eppendorf tubes.

39 Remove storage solution by spinning them into a microcentrifuge at **1500 x g, 23°C, 00:01:00** .

40 The storage solutions for the columns will have passed through and the chromatography resin will now be compacted.

41 Discard the caps, storage buffers, and place a vertical mark where resin is slanted upwards.

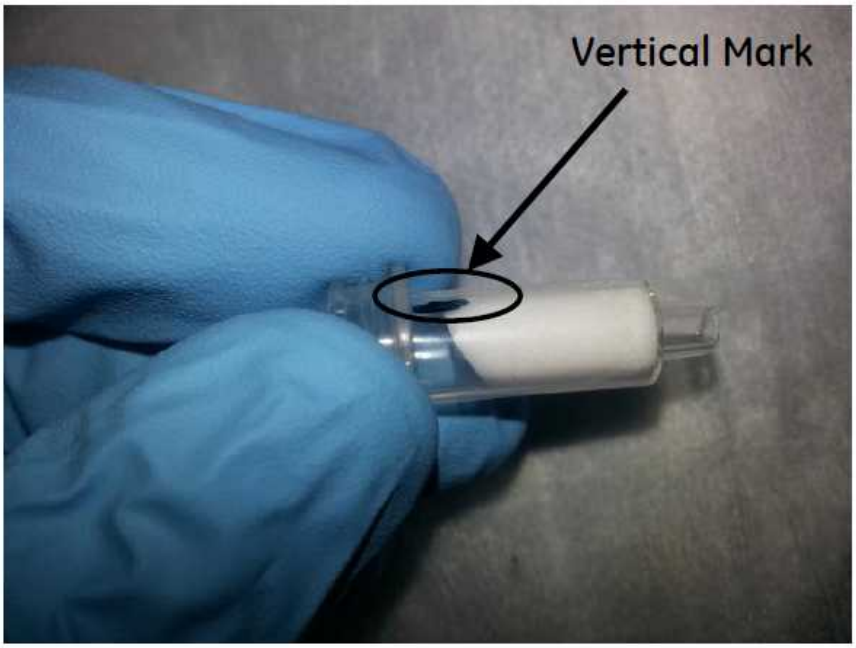

Figure 4.1: Vertical Mark On Resin Slant

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42 Add **300 µl fresh 1X PBS solution** to columns.

43 Place the columns back in the microcentrifuge. Ensure the marked side is facing towards the outside of the centrifuge.

44

Centrifuge at **1500 x g, 00:01:00** .

45 Transfer prepared columns to new, clean eppendorf tubes labeled with the antibody name.

Purifying the Direct Conjugate to Remove Unbound Dye via Zeba Spin Columns

46 If needed, add fresh 1X PBS to the conjugation reaction after the 30 minute incubation period is over so that the total volume of the conjugation reaction is > **70 µl** .

47 Apply the conjugation reaction solution to the center of one of the Zeba spin columns. Be sure not to disturb the surface of the compacted resin. Refer to Figure 5.1.

The minimum volume that can be loaded into a zeba column is **70 µl** .

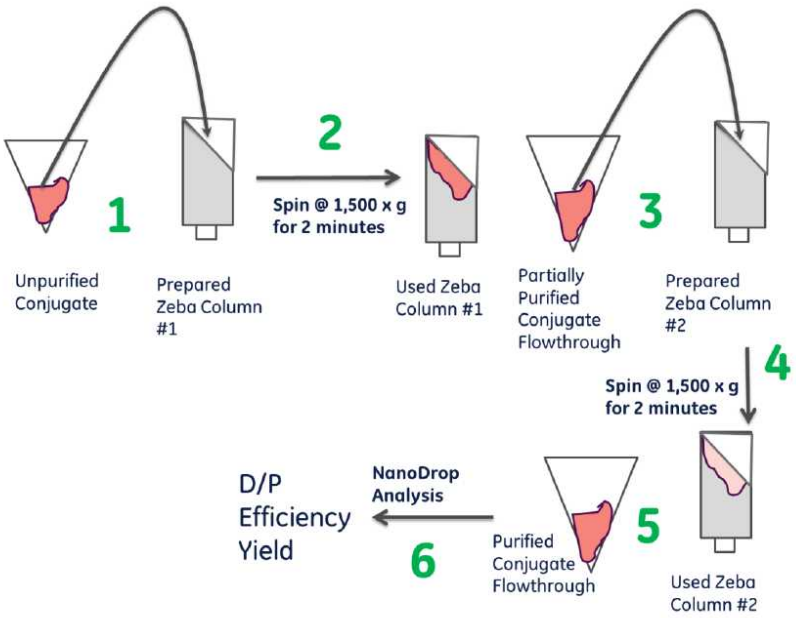

Figure 5.1: Direct Conjugate Purification Workflow

Citation: Anup Sood, Eric Williams, Liz McDonough (01/27/2021). Cell DIVEÃçÂÂç Platform | Ab Conjugation:ÃÂ Initial Conjugation & Scale up Conjugation. https://dx.doi.org/10.17504/protocols.io.bp55mq86

48 After the sample has been put on the column measure **15 µl fresh 1X PBS** and apply to the top of the column. This is referred to as a stacker and will help in overall antibody yield.

49 

Spin in the microcentrifuge at **1500 x g, 23°C, 00:02:00** . Be sure the black line on the column is facing outward.

50 The purified direct conjugate will flow through the column and collect in the bottom of the 1.5 mL eppendorf tube.

51 Remove the purified DC from the first column tube and place the sample onto the center of the second prepared column, be sure not to apply sample to the side of the compacted resin.

52 

Spin in the microcentrifuge at **1500 x g, 23°C, 00:02:00** .

This is to ensure complete removal of any unbound dye.

53 Discard the used Zeba columns and proceed to quantitative assessment.

Quantitative Assessment of Pilot Direct Conjugate via NanoDrop2000c

54 Open the NanoDrop 2000c software version 7.2.1.1 program located on the desktop of the NanonDrop computer

55 From the home screen select “Proteins and Labels”. If Cy7 was used, follow alternative procedure described earlier (**go to step #17**).

56 Select IgG under “Type”. (Refer to (1) on Figure 6.1)

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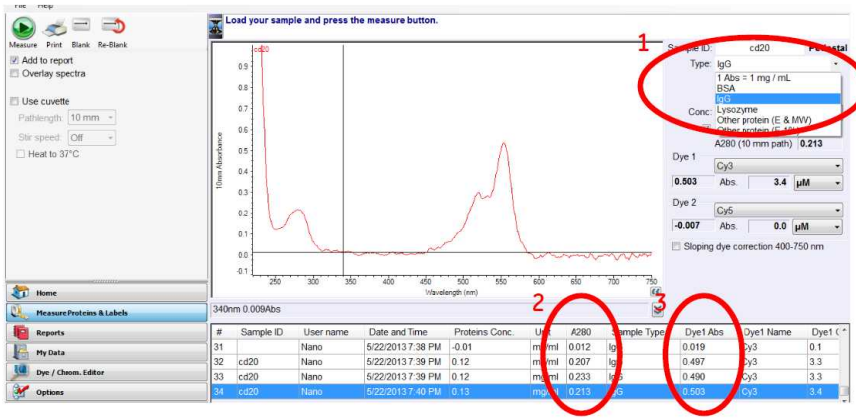

Figure 6.1: Quantitative Assessment of Direct Conjugates

57 Select dye under Dye 1: Refer to (1) of Figure 1.2 (**go to step #6**).

58 Measure **2 µl fresh 1X PBS** and place on NanoDrop pedestal to blank the instrument.

59 Select “Blank.”

60 After the measurement completes wipe the pedestal with a Kimwipe.

61 Measure **2 µl fresh 1X PBS** and place on NanoDrop pedestal to measure the baseline.

62 Select “Measure.”

63 After the measurement completes wipe the pedestal with a Kimwipe.

64 Refer to Figure 1.3 (**go to step #13**): Ensuring a Stable Baseline on the NanoDrop 2000c.

- If the baseline is poor, repeat **go to step #58**

- A Stable Baseline should read and Absorbance between 0.02 and -0.02, with no tilt in the horizontal line.
- An Unstable Baseline will look tilted or veer outside of the acceptable -0.02 and +0.02 acceptable range.

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65 Measure the purified direct conjugate **3 times**, verify the A_{280} and A_{Dye}.

Refer to Figure 6.1.

- (2) is the protein absorbance at 280nm.
- (3) is the dye absorbance at the dye specified wavelength.

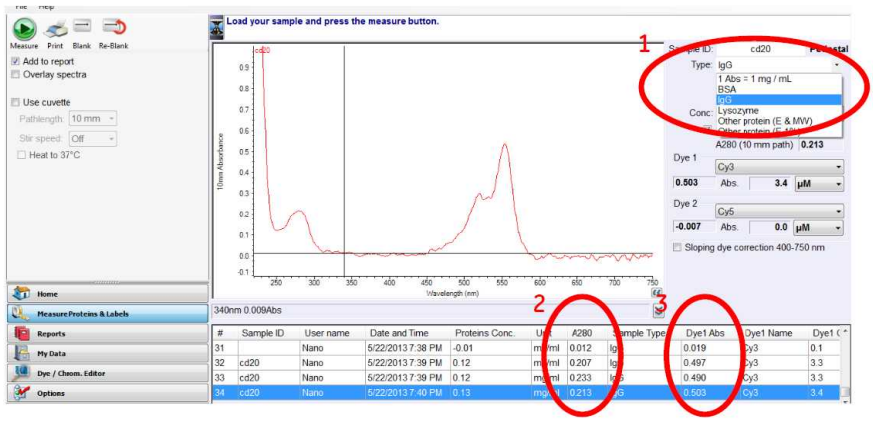

Figure 6.1: Quantitative Assessment of Direct Conjugates

66 Calculate the direct conjugate D/P and efficiency. Use the appropriate form based on the dye conjugated#

- Form-2:Cy3 Direct Conjugate
- Form-3:Cy5 Direct Conjugate
- Form-5: Cy2 Direct Conjugate
- Form-6: Cy7 Direct Conjugate
- Refer to Figure 8.6.2.

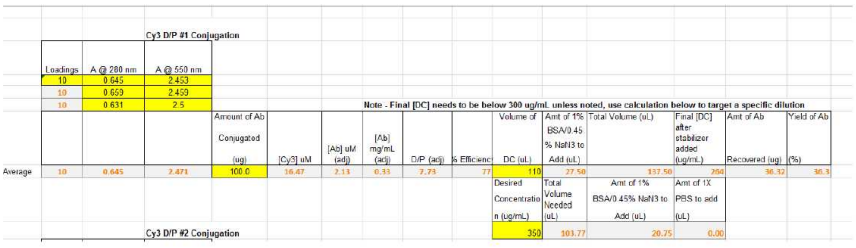

Figure 6.2: Pilot Direct Conjugate Dye To Protein Calculation

67 Take a volumetric reading of the DC with a p200 pipette.

68 Input the volume of the DC into “Volume of DC (uL)” on the D/P calculation sheet to determine the yield of the direct conjugate.

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69 Ideally, the two conjugates should be separated by at least 1.5 D/P. If they are not, a new conjugate should be made utilizing the pilot efficiency to obtain the acceptable D/P levels.

70 The acceptable range of D/P is listed on Table 2.1 (**go to step #20**) for each dye. If the conjugates fall outside of this range consult the study coordinator to determine if the conjugate should be tested, or a new lot of conjugate should be made.

Stabilizing the Conjugations

71 Refer to the appropriate D/P calculation form depending on what dye was used.

72 The template will calculate the direct conjugate concentration and amount of BSA/azide stabilizing solution to add.
- Add the stabilizing solution to the conjugation solution and mix thoroughly. Centrifuge briefly to remove liquid from the underside of the cap.

73 The Desired Concentration will be calculated on the D/P Calculation Sheet.

> Please note that the conjugates should be at a concentration < **300 Mass Percent** , adjust with calculated amount of fresh 1X PBS and stabilizing solution to reach appropriate concentration.

74 Transfer the stabilized DC to an amber tube and store at **2 °C** - **8 °C** .

75 Label the direct conjugate tubes with: biomarker name, dye, D/P, concentration, date of conjugation and vial #.

76 Input the conjugate into your reagent management system, if any.

77 Complete the Direct Conjugate Certificate of Analysis (see following section).

Complete Direct Conjugate Certificate of Analysis

78 **Antibody Information**

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Target	
Vendor	
Catalog	
Lot	

Conjugate Information

Dye #1	Cy3		
Lot Information	Lot 1	Lot 2	Lot 3
Lot#			
D/P			
Concentration	ug/mL	ug/mL	ug/mL
Staining Concentrations to be Tested per lot			
Manufacture Date			
Expiration Date			
Formulation	Aqueous buffer contains 0.2% BSA. 0.09% Sodium Azide		
Temperature Limitation	Store at 2-8°C		
Dye #2	Cy5		
Lot Information	Lot 1	Lot 2	Lot 3
Lot #			
D/P			
Concentration	ug/mL	ug/mL	ug/mL
Staining Concentrations to be Tested per lot			
Manufacture Date			
Expiration Date			
Formulation	Aqueous buffer contains 0.2% BSA. 0.09% Sodium Azide		
Temperature Limitation	Store at 2-8°C		

Controls

Batch Record Verification: Conjugates are between Cy3 **3.0** and **6.5** D/P: ☐ Yes ☐ No (check one)
Cy3 **1.5** and **4.5** D/P: ☐ Yes ☐ No (check one)

Performance Verification: Conforms? ☐ Yes ☐ No (check one)

Representative Image(s)

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We confirm that this product has been verified and conforms to previously outlined QC measures

Release Date:	
Verified By Name:	
Verified By Signature:	

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