

FITC & RITC FROM BSL

1. Use saturated ammonium sulphate (SASat + 4°C) to give a final concentration of 40% SAS for rabbit or ~~43%~~ ⁵⁰ SAS for mouse.
2. Add SAS dropwise with stirring at + 4°C. Allow to stand for 1 hour. (May be done in high speed centrifuge tube).
3. Spin at 10,000 RPM for 15 min; discard supernatant.
4. Redissolve ppt. in PBS or saline (9 gm/liter) and reprecipitate with SAS, conditions as above in #2. .9% Saline
5. Spin as in #3.
6. Redissolve ppt. in saline to approx 1/3 original serum volume.
7. Dialyze overnight at + 4°C against saline. *PBS is better.*
8. Spin at 10,000/10 min to remove denatured protein.
9. Determine protein concentration ($\frac{\text{mg/ml}}{1.4} = \text{OD}_{280}$)
10. Bring to 10 mg/ml protein in pH ~~9.0~~ ^{9.3} $\text{Na}_2\text{CO}_3 - \text{NaHCO}_3$ Buffer 0.5 M.
 [8 ml Na_2CO_3 0.5M (5.3 gm/100 ml dist H_2O) + 17 ml NaHCO_3 0.5 M (4.2 gm/100 ml dist H_2O)]. *OR 1.7 gm Na_2CO_3 and 2.86 gm NaHCO_3 in 100 ml deionized H_2O pH=9.5*
FITC 20 μ /mg protein RITC 30 μ /mg protein ~~2 1/2 hrs~~
11. Add FITC or RITC at ~~1 mg/10 mg~~ ^{25 μ /mg} protein and stir at room temperature for 1 1/2 hrs ~~-Rn~~ or overnight (16 hrs) at + 4°C. Go to next step without delay. *in dark*
12. Run Sephadex G25 (fine) column to remove free fluorescein pH 7.6 0.01 M Tris/0.01 M NaCl buffer. Pool all tubes in first peak having an OD_{280} reading greater than 0.1.
OR treat as in Step # 1,2,3 then dialyze against 0.01 M Tris/ 0.01 M NaCl pH 7.6. Using stock solutions of (A) Tris hydroxymethyl aminomethane (Sigma 7-9) and (B) HCl both at 0.2 M.
 Use 50 ml A and 43 ml B dilute to 500 ml in dist. H_2O .
 Mix equal volume of above buffer with 0.02 M Saline (1.17 gm/liter) = 0.01 M Tris/0.01 M Saline pH 7.6 and for saline gradient 0.01 M Tris/0.01 M NaCl-- 0.01 M Tris/0.5 M NaCl.
 Mix equal volume of above Tris buffer with 1.0 M saline (5.84 gm/100 ml)...
 4 hrs dialysis is plateau time for 3-5 ml sample to get rid of unreacted FI.
13. Use saline gradient explained above to run material on a DE 52 column (protein to bed ratio ~~20~~ ²⁰ OD_{280} / 1 ml packed bed). Gradient equals a total of 200-300 ml, 3 ml fractions are collected. 36 drop/tube \approx 2.3 ml
14. Spectrophotometer readings at 280 for protein, 495 for FITC or 515 for RITC are taken for each fraction.
 To calculate F/P ratio for FITC labelling

$E_{495} \text{ FITC} = .175$

$E_{280} \text{ FITC} = .054$

A. $\text{OD}_{280} - \text{protein} \quad \text{OD}_{495} - \text{FITC}$

B. $\frac{\text{OD}_{495}}{0.175} = \frac{\text{F}}{\text{P}} / \text{ml}$

C. $\text{OD}_{280} - \left(\frac{\text{F}}{\text{P}} / \text{ml} \times 0.054 \right) = \text{OD}_{280} \text{ of protein.}$

D. $\frac{\text{OD}_{280}}{1.4} = \text{mg/ml protein}$

P.P.S. CONT.

To calculate R/P ratio for RITC Labelling, see bottom of next page.

389 mw. Fluorescein
150,000 mw golbulin

$$E. \frac{F/ml}{389} \div \frac{mg/ml \text{ protein} \times 10^3 (gm \text{ protein})}{1.5 \times 10^5} \text{ Ratio Molar} = \frac{F}{P}$$

(mw protein) → whole protein
For Fab use 5×10^4 as denominator

15. Pool fractions within the following F/P ratios. 1.5 - 2.5, 2.6 - 3.5, 3.6 - 4.5
Concentrate pools by Amicon filtration, vacuum dialysis or SAS as in Steps #1,2,3
to > 2 mg/ml.
16. Dialyze overnight vs. PBS.
17. Centrifuge to remove any ppt. 10,000/10' and millipore filter
18. Absorb with mouse liver powder (1 hr at room temp. with stirring) 100 mg/10 mg
prot.
19. Centrifuge 10,000/10 min. and millipore filter
20. Redetermine protein con and F/P ratios for each pool.
21. Aliquot; Store at + 4°C.

drop \approx 60

Green Refrigerator
BBL

Note: Wash hands before handling
each dialysis tubing.

FITC - #12008 BBL
100 mgm \$25.00

RITC - #12198 BBL
100 mgm \$32

Formula for R/P Ratios.

Obtain :- OD₂₈₀ OD₅₁₅ OD₅₅₅ ..

$$\text{protein (mg/ml)} = \frac{OD_{280 \text{ nm}} - 0.56 (OD_{515 \text{ nm}})}{1.4}$$

$$R/P = \mu M \text{ TRITC} / \mu M \text{ IgG} = \frac{OD_{555 \text{ nm}}}{\text{protein conc. mg/ml}} \times 6.6$$

[Another R/P Ratio is sometimes used $\frac{OD_{280}}{OD_{515}}$]