HuCAL® Antibodies Technical Manual
Antibody Conjugation

There are two methods for the direct detection of HuCAL antibodies without the need for secondary antibodies. One is the use of Alkaline Phosphatase (AP) fusion antibodies, e.g. the Fab-A-FH format, together with an AP detection substrate, and the other is by directly labeling the HuCAL primary antibody by chemical coupling to an enzyme or dye.

The advantage of using a labeled secondary antibody is that it works with many different primary antibodies and can enhance sensitivity. However, working with only a primary antibody is often preferred because it reduces the risk of non-specific background staining from cross-reactive secondary antibodies. It also reduces incubation times, washing steps and cost, by avoiding secondary reagents.

Conjugation of HuCAL antibodies with biotin and various fluorescence dyes works in the same way as for traditional antibodies. To date, in house testing has shown that conjugation with LYNX Rapid Conjugation Kits® delivers antibodies with excellent sensitivities, which are similar to those achieved with secondary antibodies.

Covalent conjugation uses primary amine groups present in lysine residues. Since the conjugation might take place in the binding region (CDRs) of the antibody it is recommended to confirm antigen binding first before starting more complex experiments. If binding strength is reduced or even lost, a lower ratio of labeling molecules to antibodies might help. Alternatively, switching from a monovalent to a larger antibody format i.e. Fab-A format or full IgG might be beneficial. However, only a small number of antibodies are actually affected by the labeling reaction.

Site-specific conjugation of an antibody can be achieved with our Fab formats with free cysteines at the C-terminus of the heavy chain. Conjugation via the thiol groups avoids potential conjugations in the binding regions. This format can also be used for immobilization on gold surfaces.

General Points

- Best labeling ratio (antibody:reagent) is antibody and application dependent. The ratios in the following protocols are good starting points
- It is recommended to confirm antigen binding of the conjugated antibody in a simple experiment, e.g. ELISA, before starting more complex experiments. Covalent conjugation is performed via primary amines in lysine residues and might take place in the binding region (CDRs), which can reduce the binding strength of the antibody. If binding strength is altered after conjugation, a lower labeling ratio should be tested, e.g. 1:5
- Larger antibody formats, such as Fab-A or full IgG, might be beneficial for labeling compared to the monovalent Fab format due to their larger surfaces not involved in antigen binding. However, since only a small number of antibodies are actually affected by the labeling reaction switching the format is not generally required or recommended
- Buffer recipes and recommended sources for the reagents and antibodies are listed in the appendices

Conjugation using LYNX Rapid Conjugation Kits

LYNX Rapid and Rapid Plus conjugation kits offer extremely fast and simple conjugation of antibodies and other proteins to biotin, fluorescent or enzyme labels. LYNX kits offer the following advantages:

- Easy protocol with just 30 seconds hands-on time and an incubation time of either 15 minutes with the Rapid Plus kits or 3 hours with the Rapid kits to finish the conjugation
- No purification required, so there is no loss of antibody
- High quality conjugates are ready to use
- Assay sensitivities are equal to those achieved with secondary antibodies

LYNX kits are available in a range of sizes for easy, scalable conjugations from microgram to milligram quantities. In addition, they are available for all commonly used reporter enzymes, fluorescent dyes and biotin.

In the procedure, the antibody is mixed with the labeling reagents followed by 15 minute, 3 hour or overnight incubation. The reaction is stopped and the antibody is ready to use (Figure 1). All materials and reagents are provided with the kit and no purification of the antibody is required after the labeling reaction.

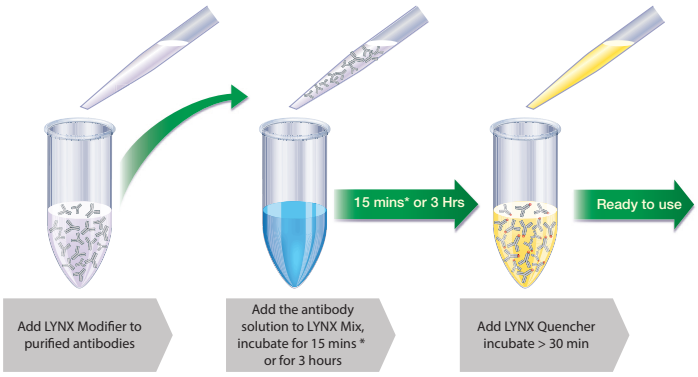

Fig.1. LYNX Conjugation Kit Procedure. *15 minute incubation only for LYNX Rapid Plus kits.

LYNX Conjugation Kits

Fluorescent dyes

Conjugates	ME/ME*	LYNX Rapid & Rapid Plus ** Kits	No. Vials/Antibody Conjugated Per Vial
DyLight® 488	***493 / 518***	***LNK221D488*** ***LNK222D488*** ***LNK223D488***	***3 / 200 µg*** ***1 / 2 mg*** ***3 / 20 µg***
Fluorescein	494 / 520	LNK061F LNK062F LNK063F	3 / 200 µg 1 / 2 mg 3 / 20 µg
Cy3 (Cyanine Dye 3)	***550 / 570***	***LNK201CY3*** ***LNK202CY3*** ***LNK203CY3***	***3 / 200 µg*** ***1 / 2 mg*** ***3 / 20 µg***
R-Phycoerythrin (RPE)	535 / 575	LNK021RPE LNK022RPE LNK023RPE LNK024RPE	1 / 60 µg 3 / 60 µg 1 / 600 µg 3 / 10 µg
Rhodamine	***544 / 576***	***LNK251RHO*** ***LNK252RHO*** ***LNK253RHO***	***3 / 200 µg*** ***1 / 2 mg*** ***3 / 20 µg***
DyLight 550	***562 / 576***	***LNK231D550*** ***LNK232D550*** ***LNK233D550***	***3 / 200 µg*** ***1 / 2 mg*** ***3 / 20 µg***
RPE/Texas Red®	535 / 615	LNK171PETR LNK172PETR LNK173PETR LNK174PETR	1 / 100 µg 3 / 100 µg 1 / 1 mg 3 / 10 µg
APC (Allophycocyanin)	650 / 662	LNK031APC LNK032APC LNK033APC LNK034APC	1 / 150 µg 3 / 150 µg 1 / 1.5 mg 3 / 10 µg
Cy5 (Cyanine Dye 5)	***643 / 667***	***LNK211CY5*** ***LNK212CY5*** ***LNK213CY5***	***3 / 200 µg*** ***1 / 2 mg*** ***3 / 20 µg***
RPE/Cy5	488 / 670	LNK081C LNK082C LNK083C LNK084C	1 / 60 µg 3 / 60 µg 1 / 600 µg 3 / 10 µg
DyLight 650	***652 / 672***	***LNK241D650*** ***LNK242D650*** ***LNK243D650***	***3 / 200 µg*** ***1 / 2 mg*** ***3 / 20 µg***
PerCp	482 / 677	LNK071PERCP LNK072PERCP LNK073PERCP LNK074PERCP	1 / 100 µg 3 / 100 µg 1 / 1 mg 3 / 10 µg
APC/Cy5.5	633 / 694	LNK121APCCY5.5 LNK122APCCY5.5 LNK123APCCY5.5 LNK124APCCY5.5	1 / 150 µg 3 / 150 µg 1 / 1.5 mg 3 / 10 µg
PerCp/Cy5.5	482 / 700	LNK141PERCPCY5.5 LNK142PERCPCY5.5 LNK143PERCPCY5.5 LNK144PERCPCY5.5	1 / 100 µg 3 / 100 µg 1 / 1 mg 3 / 10 µg
RPE/Cy5.5	535 / 694	LNK091PECY5.5 LNK092PECY5.5 LNK093PECY5.5 LNK094PECY5.5	1 / 60 µg 3 / 60 µg 1 / 600 µg 3 / 10 µg
APC/Cy7	633 / 776	LNK131APCCY7 LNK132APCCY7 LNK133APCCY7 LNK134APCCY7	1 / 150 µg 3 / 150 µg 1 / 1.5 mg 3 / 10 µg
RPE/Cy7	535 / 776	LNK111PECY7 LNK112PECY7 LNK113PECY7 LNK114PECY7	1 / 60 µg 3 / 60 µg 1 / 600 µg 3 / 10 µg

Bold Denotes Rapid Plus Kits

Enzymatic dyes

Conjugates	LYNX Rapid Kits	No. Vials/Antibody Conjugated Per Vial
Alkaline Phosphatase (AP)	LNK011AP LNK012AP LNK013AP LNK014AP	1 / 100 µg 3 / 100 µg 1 / 1 mg 3 / 10 µg
Glucose Oxidase	LNK051GOX LNK052GOX LNK053GOX	3 / 100 µg 1 / 1 mg 3 / 10 µg
Horseradish Peroxidase (HRP)	LNK001P LNK002P LNK003P LNK004P LNK005P LNK006P	1 / 400 µg 3 / 400 µg 1 / 4 mg 5 / 4 mg 1 / 20 mg 3 / 40 µg

Biotin and streptavidin dyes

Conjugates	LYNX Rapid Kits	No. Vials/Antibody Conjugated Per Vial
Biotin (Type 1)	LNK041B LNK042B LNK043B	3 / 200 µg 1 / 2 mg 3 / 20 µg
Biotin (Type 2)	LNK191B LNK192B LNK193B	3 / 200 µg 1 / 2 mg 3 / 20 µg
Streptavidin	LNK161STR LNK162STR LNK163STR	3 / 100 µg 1 / 1 mg 3 / 10 µg

*Maximum excitation (nm) / Maximum emission (nm)

** Only 15 minute incubation time required

Antibodies conjugated with LYNX kits offer excellent sensitivities and therefore can often be used at lower concentrations than needed with typical secondary reagents. Conditions that yield the best signal with low background need to be optimized for each antibody. For many assays, we recommend diluting the conjugated antibody in HISPEC assay diluent (BUF049) in order to obtain superior results.

Visit **bio-rad-antibodies.com/lynx** for more information.

HRP Conjugation of Small Amounts of Antibodies

For antibody characterization, usually only small amounts of conjugated antibody are required for a large number of antibodies. For this purpose we have adjusted the volume of antibody in the Lynx Rapid HRP conjugation kit protocol.

Procedure

1. For antibodies with high concentration, adjust to 1mg/ml by adding PBS. To achieve optimal results concentration should not be below 0.5 mg/ml.
2. To 100 µl of antibody add 10 µl of LYNX Modifier reagent (i.e. 1 µl of Modifier for every 10 µl antibody).
3. Dissolve lyophilized LYNX reagent (LNK003P or LNK004P) in 300 µl PBS. The dissolved reagent is not stable and has to be used immediately.
4. Depending on your antibody format add the required amounts of LYNX HRP reagent (Table 1) to your antibody and mix. A 1:1 molar ratio antibody:HRP reagent is usually sufficient.
5. Incubate for 3 h at room temperature (RT).
6. Add 10 µl of LYNX Quencher reagent (i.e. 1 µl of Quencher for every 10 µl of antibody).
7. Incubate for 30 min at RT.
8. Calculate the new antibody concentration:
   $c = c_0 * (V_0/V_{total})$
   c_0: antibody start concentration
   V_0: antibody start volume
   V_{total}: total end volume

This is the concentration of the antibody not taking the weight of the HRP into consideration.

Table 1. Recommended amounts of LYNX HRP reagent for different antibody formats

Antibody Format	Amount HRP Reagent Ab:HRP 1:1 to 1:4
Fab	22-87 µl
Fab-dHLX	10-39 µl
Fab-A	6-23 µl
IgG	7.5-30 µl

Example

Western blotting

An anti-cyclophilin A antibody in the Fab-A-V5Sx2 format was used in Western blot analysis of HEK293 cell lysate, either after direct HRP conjugation with a LYNX kit (LNK001P) or in combination with an HRP conjugated anti-V5 tag secondary antibody (MCA1360P) (Figure 2). The labeled antibody was diluted in HISPEC assay diluent (BUF049) and the blot was developed with a chemiluminescent detection reagent.

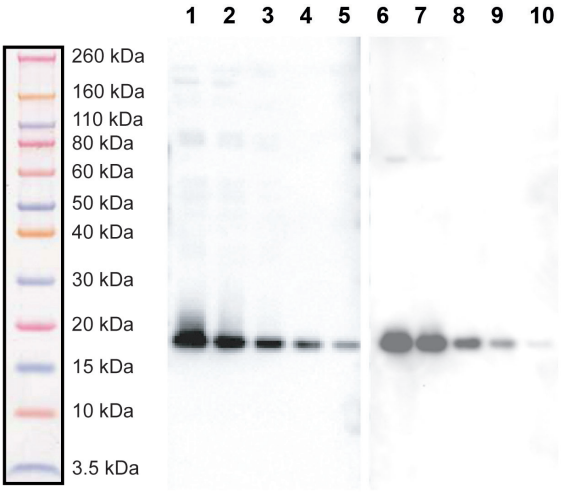

Fig. 2. Western blot Comparison of LYNX HRP Conjugated Antibody vs. Typical Secondary Antibody. Lanes 1-5, Serial dilutions of HEK293 cell lysate detected with LYNX HRP conjugated anti-cyclophilin A. **Lanes 6-10,** The same samples detected with unconjugated anti-cyclophilin A and an anti-V5:HRP secondary antibody.

Conjugation to FITC, Cy5, and Biotin using Conventional Kits

Important points before starting

- The appropriate quantity of reagent dye depends on the antibody, its concentration and on desired levels of labeling
- For most situations, a 10 to 20-fold molar excess of the reagent dye is recommended; this will label each antibody with an average of 2-4 dye molecules
- If lower levels of labeling are required, use a 5 to 10 fold molar excess of reagent dye
- For antibody concentrations below 0.5 mg/ml, an approximately 20 to 30-fold molar excess of reagent dye is recommended
- Sample calculations for the required molar excess of reagent dye are provided for each procedure

Conjugation with Fluorescein Isothiocyanate (FITC)

FITC labeling of antibodies can be performed using commercially available kits. The protocol described on page 4 uses the kit supplied by Thermo Fisher Scientific, and follows the manufacturer's instructions. It describes the conjugation of 1 mg of a bivalent and monovalent antibody, and can be scaled down to provide labeling of smaller amounts.

Table 2. Calculation of reagent dye volumes, FITC

	Mol. Weight	nmol in 1 mg Reagent	nmol in 12-fold Molar Excess of Reagent	Required Volume of Reagent
FITC	389 Da	2500 nmol/ml (solution of 1 mg/ml)	-	-
Fab	~50 kDa	20 nmol	12x20 nmol = 240 nmol	240 nmol of a FITC reagent solution at 2500 nmol/ml = 96 µl
Fab-dHLX	~120 kDa	8 nmol	12x8 nmol = 96 nmol	96 nmol of a FITC reagent solution at 2500 nmol/ml = 38 µl
Fab-A	~200 kDa	5 nmol	12x5 nmol = 60 nmol	60 nmol of a FITC reagent solution at 2500 nmol/ml = 24 µl

Procedure

1. Prepare a fresh solution with 1 mg of FITC in 10 ml PBS.
   The solution is stable for several hours. Higher temperatures, traces of amines (e.g. Tris), and non-neutral pH greatly reduce the stability. Minimize exposure to light, as FITC is light sensitive.
2. For **bivalent** Fab labeling, add 38 µl (Fab-dHLX format) or 24µl (Fab-A format) reagent solution to 1 mg antibody.
   For **monovalent** Fab labeling (Fab format), add 96 µl reagent solution to 1 mg antibody.
   Antibody concentration should be between 0.5 and 2 mg/ml. Lower concentrations of antibody require a higher reagent excess (see 'Important points before starting').
3. Incubate for 30 minutes at RT, or on ice for 2 hours.
4. Follow the purification protocol described on page 5 .

Conjugation with Cy5

Table 3. Calculation of reagent dye volumes, Cy5

	Mol. Weight	nmol in 1 mg Reagent	nmol in 12-fold Molar Excess of Reagent	Required Volume of Reagent
Cy5-NHS		6300 nmol/ml (solution of 5 mg/ml)	-	-
Fab	~50 kDa	20 nmol	12x20 nmol = 240 nmol	240 nmol of a Cy5 reagent solution at 6300 nmol/ml = 38 µl
Fab-dHLX	~120 kDa	8 nmol	12x8 nmol = 96 nmol	96 nmol of a Cy5 reagent solution at 6300 nmol/ml = 15 µl
Fab-A	~200 kDa	5 nmol	12x5 nmol = 60 nmol	60 nmol of a Cy5 reagent solution at 6300 nmol/ml = 10 µl

Procedure

1. Prepare a fresh solution of 5 mg of Cy5 mono NHS ester in 1 ml dimethylsulfoxide (DMSO).
   The solution is stable for several hours. A fresh solution should be prepared for each experiment since DMSO is hygroscopic and traces of water will greatly reduce the stability of the solution. Minimize exposure to light, since Cy5-NHS is light sensitive.
2. For **bivalent** Fab labeling, add 15 µl (Fab-dHLX format) or 10 µl (Fab-A format) reagent solution to 1 mg antibody.
   For **monovalent** Fab labeling (Fab format), add 38 µl reagent solution to 1 mg antibody.
   Antibody concentration should be between 0.5 and 2 mg/ml. Lower concentrations of antibody require a higher reagent excess (see 'Important points before starting').
3. Incubate for 30 minutes at RT, or on ice for 2 hours.
4. Follow the purification protocol described on page 5.

Conjugation with Biotin

Conjugation with biotin can be performed using the EZ-link Sulfo-NHS-LC-Biotin.

Table 4. Calculation of reagent volumes, Biotin

	Mol. Weight	nmol in 1 mg Reagent	nmol in 12-fold Molar Excess of Reagent	Required Volume of Reagent
Biotin-NHS		1800 nmol/ml (solution of 5 mg/ml)	-	-
Fab	~50 kDa	20 nmol	12x20 nmol = 240 nmol	240 nmol of a biotin reagent solution at 1800 nmol/ml = 133 µl
Fab-dHLX	~120 kDa	8 nmol	12x8 nmol = 96 nmol	96 nmol of a biotin reagent solution at 1800 nmol/ml = 53 µl
Fab-A	~200 kDa	5 nmol	12x5 nmol = 60 nmol	60 nmol of a biotin reagent solution at 1800 nmol/ml = 33 µl

Procedure

1. Prepare a fresh solution with 1 mg of EZ-link Sulfo-NHS-LC-Biotin (Biotin-NHS) in 1 ml PBS.
   The solution is stable for several hours. High temperatures, traces of amines (e.g. Tris), or non-neutral pH greatly reduce the stability of the solution.
2. For **bivalent** Fab labeling, add 53 µl (Fab-dHLX format) or 33 µl (Fab-A format) reagent solution to 1 mg of antibody.
   For **monovalent** Fab labeling (Fab format), add 133 µl reagent solution to 1 mg antibody.
   Antibody concentration should be between 0.5 and 2 mg/ml. Lower concentrations of antibody require a higher reagent excess (see 'Important points before starting').

3. Incubate for 30 minutes at RT, or on ice for 2 hours.
4. Follow the purification protocol on page 5.

Purification

For each type of labeling (when a LYNX kit is not used), there are three options for separating the antibody from the unreacted reagent.

Purification on a Econo-Pac® 10DG column or comparable SEC column

1. Add 50 µl 1 M Tris, pH 7.4, to stop the reaction (this inactivates NHS esters), and use the resulting solution for purification.
2. Equilibrate the column twice with 8 ml 3x PBS.
3. Adjust the reaction volume to 2 ml using 3x PBS.
4. Apply 2 ml reaction mix to the column and discard the flow-through.
5. Wash the column using 1 ml 3x PBS and discard the flow-through.
6. Elute with 3 ml 3x PBS.

Note: The antibody yield is approximately 80% of the starting amount.

Dialysis using 3x PBS

1. Add 50 µl 1 M Tris, pH 7.4, to stop the reaction (this inactivates NHS esters).
2 Perform two dialysis steps using at least a 50-fold excess of dialysis volume. Slide-A-Lyzer® cassettes (0.5-3 ml) with a molecular weight cut-off (MWCO) of 10,000 are recommended

Dialysis for Small-scale Reactions

1. Add 50 µl 1 M Tris, pH 7.4, to stop the reaction (this inactivates NHS esters).
2 Perform one dialysis step using 3x PBS using at least a 200-fold excess of dialysis volume. The Mini Slide-A-Lyzer with a MWCO of 3,500 is recommended.

Note: For monovalent Fab fragments, 1x PBS can also be used as dialysis buffer.

Example

Biotinylation

Successful biotinylation of a mono- and bivalent HuCAL antibody against NFκB was shown by ELISA. The biotinylated antibody was immobilized on a neutravidin plate and detected with an anti-Fab antibody conjugated to AP and AttoPhos® substrate (Figure 3).

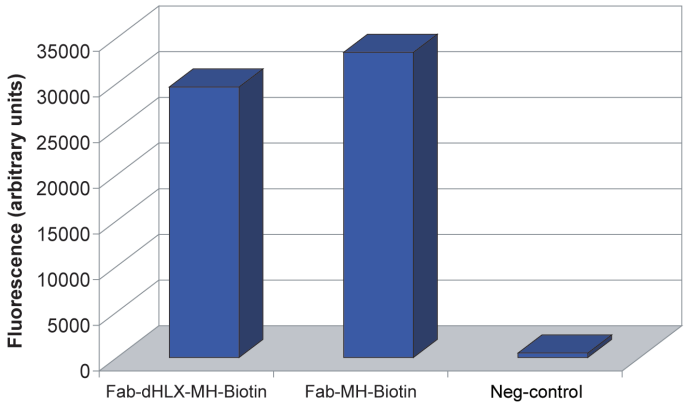

Fig. 3. ELISA Detects Biotinylated HuCAL Fab. Immobilization of biotinylated Fab on neutravidin followed by detection with an anti-Fab polyclonal antibody. The negative control is the same HuCAL Fab without biotinylation.

Troubleshooting

Problem	Possible Cause and Course of Action
Failed labeling reaction	1. Incompatible buffer used. Use a buffer that does not contain primary amines or SH groups. 2. Reagent hydrolyzed and non-reactive. Prepare labeling reagent immediately before use, and do not store the reagent in aqueous solution. 3. Reagents expired or not stored correctly. Use fresh reagents and store according to the manufacturer's instructions.
Fluorescence signal too low	1. Too few or too many dye molecules coupled to the antibody. Use a higher or lower ratio of antibody to dye (see 'Important points before starting'). 2. Buffer contains additives which interfere with the labeling reaction (e.g. thiomersal). Dialyze the antibody and try it again. 3. Antibody was conjugated in the CDR region which interferes with antigen binding. Test different antibodies or use lower amount of labeling reactant.

Appendices

Buffer composition

Buffer	Composition	Storage
PBS	136 mM NaCl 2.68 mM KCl 8.1 mM Na_2HPO_4 1.46 mM KH_2PO_4	Room temperature

Source of reagents

Reagent	Supplier	Catalog number
BSA	Sigma	A7906
Cy5 Mono NHS Ester	GE Healthcare	PA15100
Econo-Pac 10DG Columns	Bio-Rad	732-2010
EZ-link sulfo-NHS-LC-Biotin	Thermo Fisher Scientific	21335
FITC labeling reagents	Thermo Fisher Scientific	46410
HISPEC Assay Diluent	Bio-Rad	BUF049
LYNX Rapid HRP Conjugation Kit	Bio-Rad	LNK001P
Mini slide-A-Lyzer, MWCO 3,500	Thermo Fisher Scientific	69550
PBS	Thermo Fisher Scientific	14190-094
Slide-A-Lyzer, 0.5-3 ml, MWCO 10,000	Thermo Fisher Scientific	66380

Negative control antibodies

Refer to section 'Reagents to Support HuCAL Assay Development' for a list of negative control antibodies corresponding to each of the available Fab and full immunoglobulin formats. Negative controls are specific for green fluorescent protein (GFP) that does not exist in mammalian cells.

Technical Assistance

A group of experienced technical advisors are available to help with any questions regarding HuCAL antibodies and their applications, both before project start and after antibody delivery. Since HuCAL technology provides many opportunities for optimizing the antibody generation process according to customer needs we encourage the discussion of your antibody project with one of our scientists at an early stage. They can help with project design, antigen preparation, and provide information about the many options that are simply not available with typical animal-based technologies. Once the antibodies are delivered, they can also provide valuable advice about protocols and detection systems.

Contact us at antibody_sales_muc@bio-rad.com or visit bio-rad-antibodies.com/HuCAL for more information. General technical support requests related to catalog antibodies and accessory products can also be addressed to antibody_tech_uk@bio-rad.com.

Confidentiality

We treat all data and material provided by customers as completely confidential, including customer name and affiliation. Information from customers is used only as necessary for the successful and safe performance of the antibody generation project. We are pleased to provide a signed Confidential Disclosure Agreement (CDA) upon request.

Visit **bio-rad-antibodies.com** for more information.

BIO-RAD

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