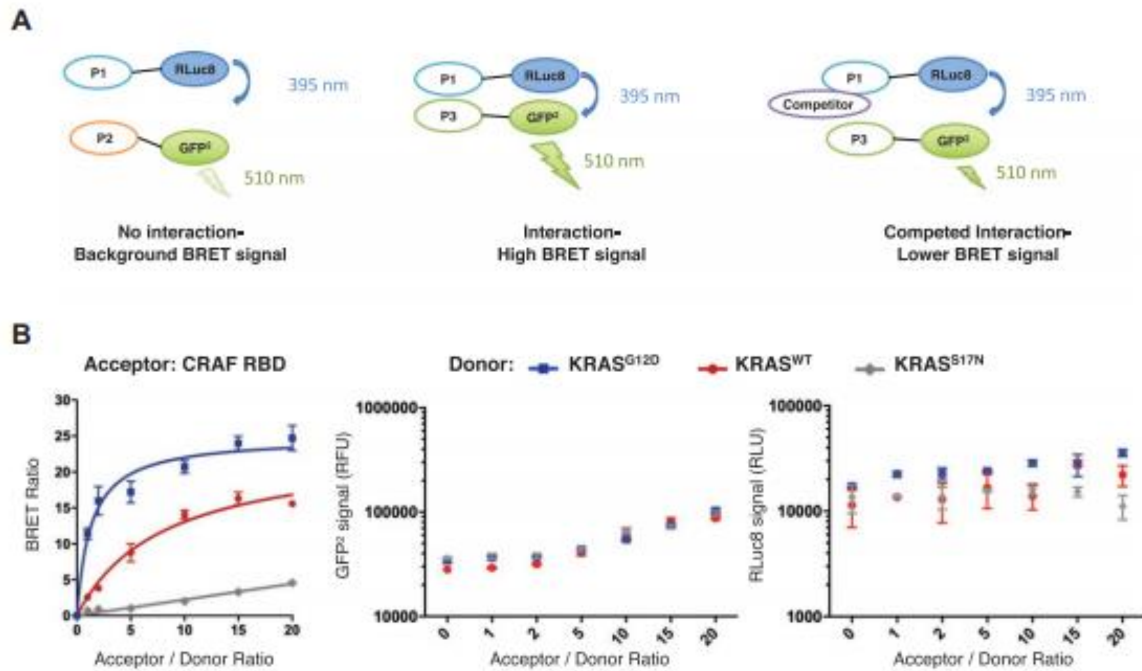


Figure 1 Use of the BRET-based RAS biosensors to assess a PPI. (A) A schematic representation of the BRET-based biosensors. A protein (P1) is fused to the donor moiety RLuc8 and a protein (P2 or P3) is tagged with the acceptor moiety GFP2. If P1 does not bind to P2, it only produces a background BRET signal. However, when P1 interacts with P2, it induces a BRET signal, if the RLuc8 and GFP2 domains are within 10 nm. This BRET signal can be decreased by addition of a competitor (either by a macromolecule or a small molecule inhibitor). (B) A representative donor saturation assay between KRASG12D, KRASWT and KRASS17N (donors) and CRAF RBD (acceptor) with total GFP2 and RLuc8 controls. Where error bars are presented, these correspond to mean values ± SD of quadruplicates technical repeats.

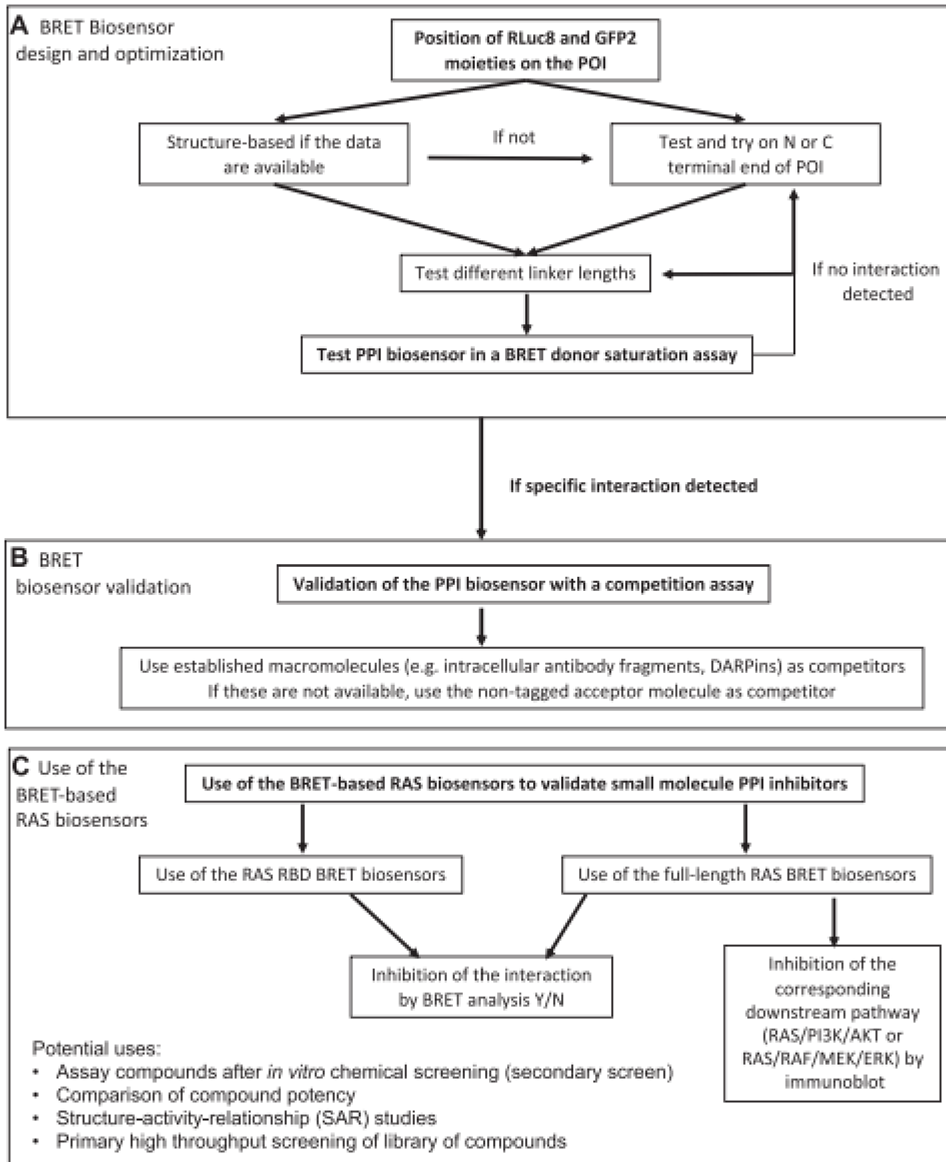


Figure 2 Development of BRET-based PPI biosensors and application flow chart. The BRET2- based PPI interaction assay can be used for the detection and quantification of interactions between two proteins in mammalian cells and their inhibition by macromolecules or small molecules. The figure shows the general requirements for establishing the assay (panel A). The BRET competition assay is used to show the validation requirements of the BRET biosensors (panel B). Examples of utility of the BRET-based RAS biosensors are shown for studying RAS-effector interactions inhibition by small molecules (panel C). POI = protein of interest.

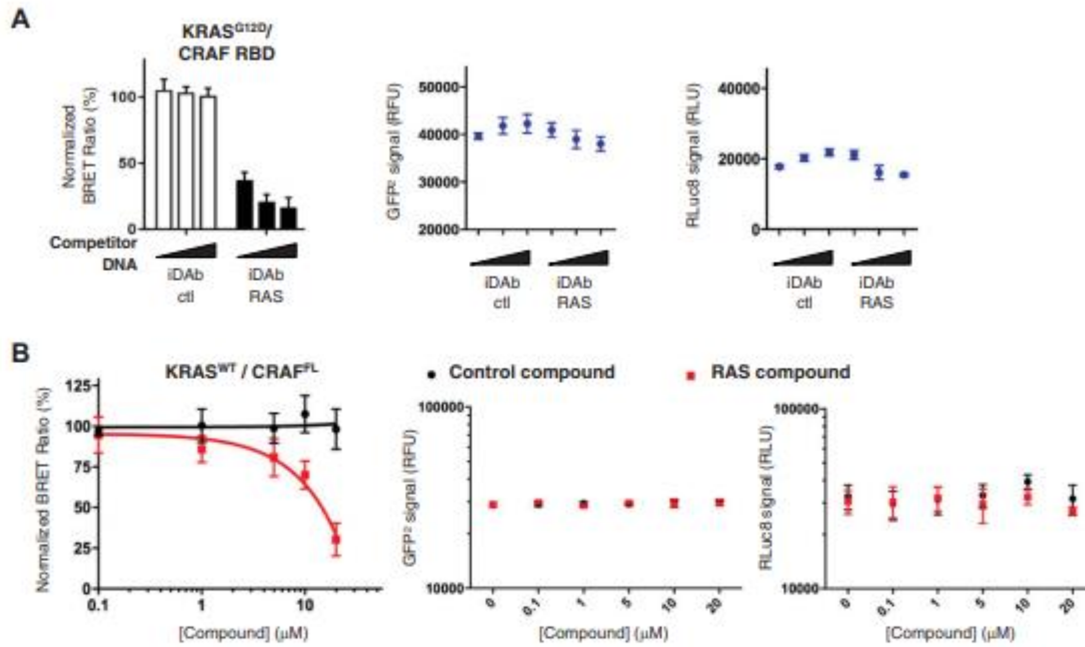
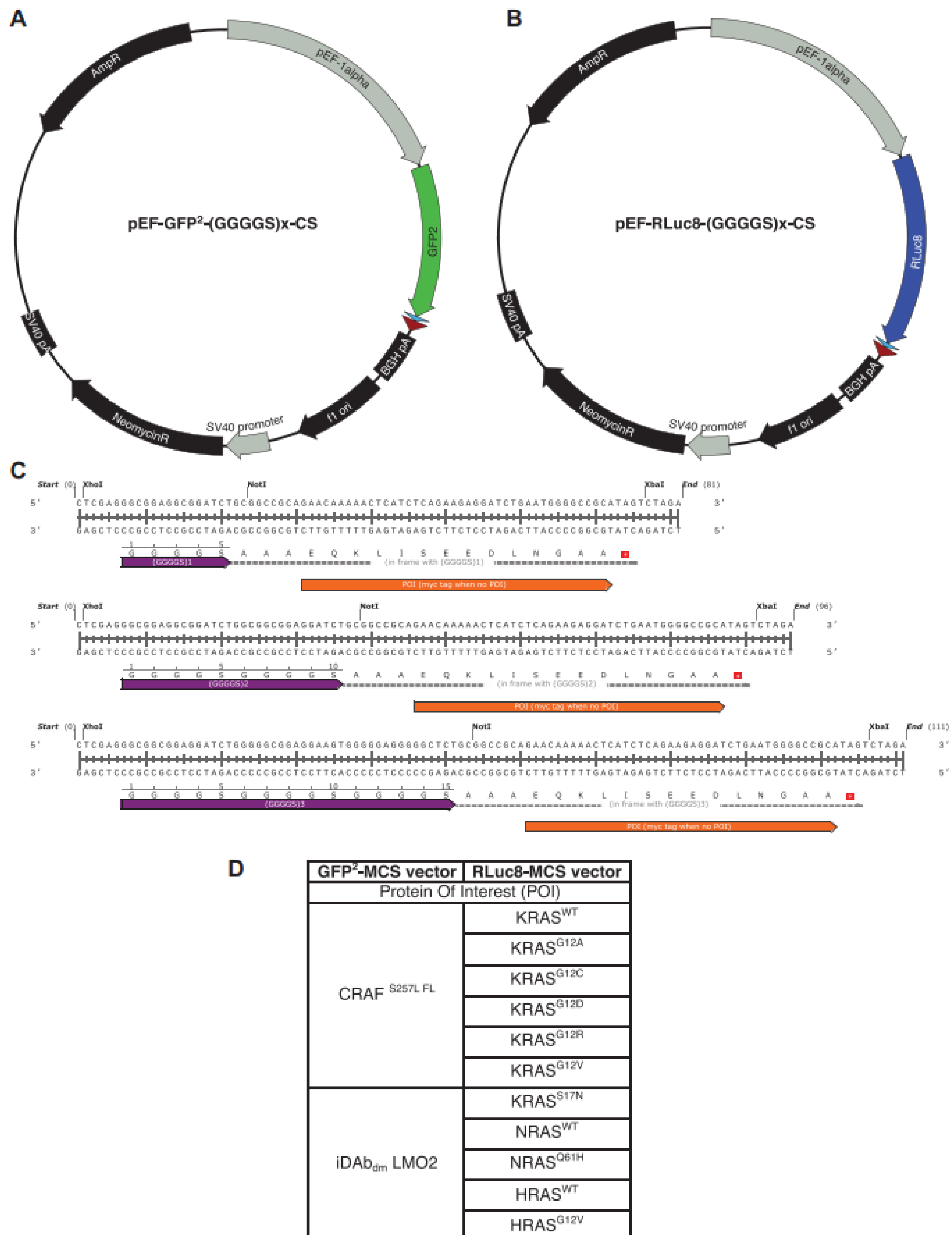


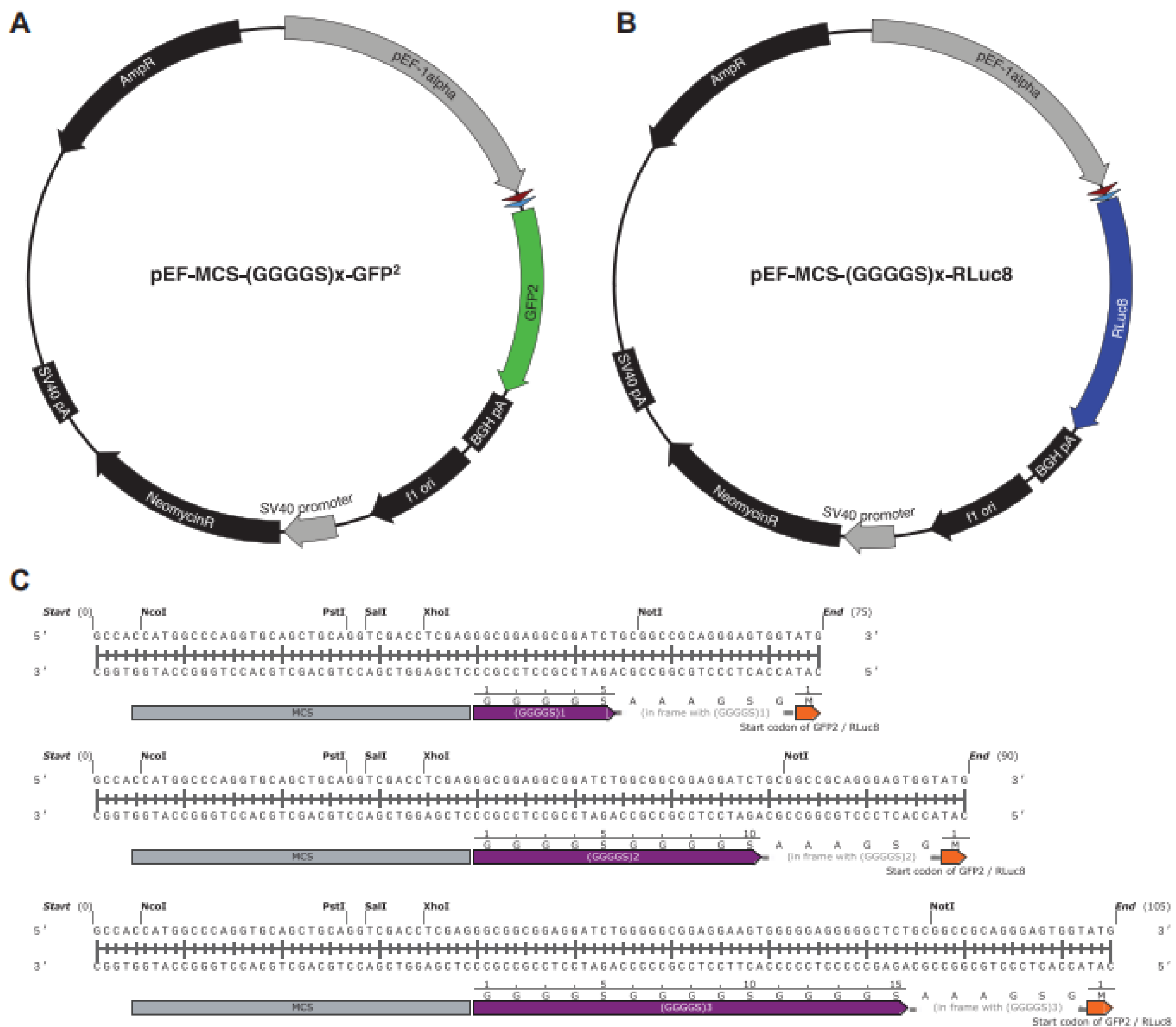
Figure 3 Use of the BRET-based RAS biosensors in competition assays. (A) A BRET competition assay between KRASG12D/CRAF RBD and intracellular domain antibodies (iDAbs). The non-relevant anti-LMO2 iDAb (called hereafter iDAb control, ctl) is a negative control and the anti-RAS iDAb (herein named iDAb RAS) is a positive control. Total GFP2 and RLuc8 levels from the competition assay are also shown. (B) A BRET competition assay with concentration response curves (0.1; 1; 5; 10 and 20 µM of compounds) showing the effect of a negative compound (black circles) and an anti-RAS compound (red squares) on KRASWT/CRAFFL interaction after EGF treatment (5 min at 50 ng/ml). Total GFP2 and RLuc8 signal are shown. The error bars correspond to mean values ± SD of quadruplicates technical repeats.



GFP²-MCS vector	RLuc8-MCS vector
Protein Of Interest (POI)	
CRAF $^{S257L\ FL}$	KRAS^{WT}
	KRAS^{G12A}
	KRAS^{G12C}
	KRAS^{G12D}
	KRAS^{G12R}
	KRAS^{G12V}
iDAb$_{dm}$ LMO2	KRAS^{S17N}
	NRAS^{WT}
	NRAS^{Q61H}
	HRAS^{WT}
	HRAS^{G12V}

Figure 4 Maps of the BRET-based biosensor vectors with C terminal cloning site. (A, B) pEF-GFP2-(GGGGS)x-CS and pEF-RLuc8-(GGGGS)x-CS are respectively generic acceptor (A) and donor vectors (B) where the proteins of interest are cloned in C terminal of the GFP2 and RLuc8 moieties (CS: cloning site). A glycine serine linker was added between the GFP2/RLuc8 and the cloning site of the proteins of interest. Three different lengths are available: (GGGGS)1, (GGGGS)2 and (GGGGS)3. The expression of the donor and acceptor fusion proteins is under the control of the human EF1α

promoter (pEF-1alpha). Stable clones can be selected in mammalian cells with the neomycin/G418 selection marker. The plasmids contain the ampicillin resistance gene (AmpR) for bacterial selection. (C) An expanded view of the different linker DNA and amino acid sequences and the cloning site. The linkers are cloned between XhoI/NotI sites and the proteins of interest are cloned between NotI/XbaI sites (or alternatively PspOMI/XbaI). (D) Table showing the different proteins cloned into these vectors to establish the BRET-based RAS biosensors.



D

MCS-GFP² vector	MCS-RLuc8 vector
Protein Of Interest (POI)	
iDAb RAS	LMO2
iDAb$_{dm}$ RAS	
iDAb control (LMO2)	
PI3Kα RBD	
PI3Kγ RBD	
CRAF RBD	
RALGDS RA	
PI3Kα^{FL} (± p85a^{FL})	

Figure 5 Maps of the BRET-based biosensor vectors with N terminal cloning sites. (A, B) pEF-MCS-(GGGGS)x-GFP2 and pEF-MCS-(GGGGS)x-RLuc8 are respectively generic acceptor (A) and donor vectors (B) where the proteins of interest are cloned in the N terminal of the GFP2 and RLuc8 moieties in the Multiple Cloning Site (MCS). The same glycine serine linkers shown in Figure 4 were added between the MCS and the GFP2/RLuc8. (C) An expanded view of the different linker DNA and amino acid sequences and the MCS. The proteins of interest can be cloned between different sites: NcoI, PstI, SalI or XhoI. (D) Table showing the different proteins cloned into these vectors to establish the BRET-based RAS biosensors.