Ras GTPase Chemi ELISA Kit

Catalog No. 52097

(Version B4)

TABLE OF CONTENTS Page

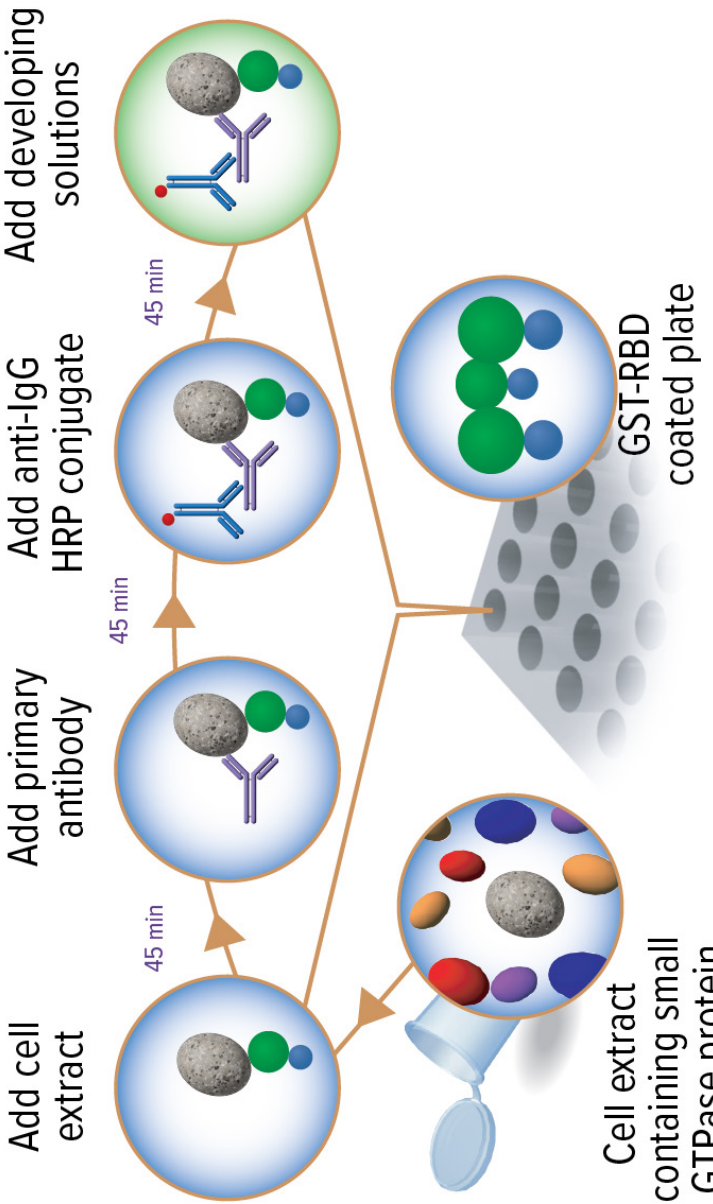
Overview

Small GTP-binding proteins (GTPases) are important regulators of signal transduction pathways. The small GTPase Ras acts as a key regulator of cellular functions including proliferation and differentiation and is also implicated in tumorigenesis, tumor invasion and morphogenesis. Oncogenic mutations in the *ras* gene are present in approximately 30% of all human cancers[1]. Because of the critical role of Ras in tumor development, it is important to be able to screen novel signaling pathways for activating Ras. Traditional methods for monitoring Ras activation, such as Western blotting, are tedious and time consuming and not suitable to high-throughput analysis.

The Ras GTPase Chemi ELISA Kit is designed specifically for the study of Ras activation and can be used to study novel signaling pathways for activating Ras or for detecting oncogenic Ras. Ras GTPase ELISA Kits contain a Raf-RBD protein fused to GST that will be coated onto the provided 96-well, glutathione-coated plate. The activated Ras contained in the cellular extract specifically binds to Raf-RBD, while inactive Ras does not bind. Bound Ras is detected by incubating with a primary antibody that detects H-Ras in mouse and H- & K-Ras in human samples. Addition of a secondary antibody conjugated to horseradish peroxidase (HRP) and developing solution provides a sensitive chemiluminescent readout that is easily quantified by luminescence. The 96-well plate is suitable for manual use or high-throughput screening applications.

product	format	catalog no.
Ras GTPase Chemi ELISA Kit	1 x 96 rxns	52097

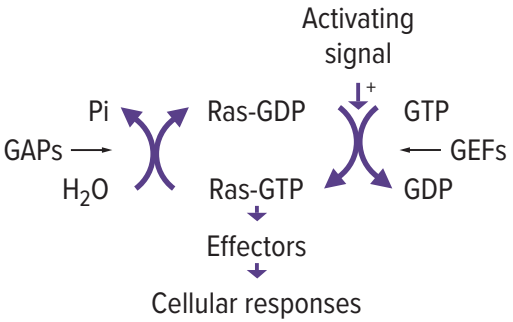
Flow Chart of Process



Introduction

Ras GTPase

GTPases (also called GTP-binding proteins) are a family of enzymes that bind to and hydrolyze GTP, allowing them to function as molecular switches. When bound to GDP, the GTPase protein is in its inactive form. Activation is controlled by regulatory proteins called guanine nucleotide exchange factors (GEFs), which induce the release of GDP. Because GTP is present in the cell in a large excess over GDP, the resulting empty nucleotide-binding site is filled by GTP and the GTPase is activated. Another class of proteins, GTPase-activating proteins (GAPs), speed up hydrolysis of GTP to GDP, inactivating the GTPase2. The figure below illustrates Ras activation.



The small GTPase Ras family regulates a variety of cell functions including proliferation and differentiation. Family members include Ras (H, K, N, R, M and TC21), Rap (1A, 1B, 2A and 2B) and Ral (A and B), and are characterized by similarities in their effector domains[3]. Ras proteins consist of about 190 amino acid residues that are highly conserved in the N and C termini. Most variations between proteins occur near the C-terminal hypervariable domain, and this variation is presumed to be responsible for differences in function4.

Activated Ras in turn activates several distinct effectors, such as the serine-threonine kinase Raf1, phosphoinositide 3´-kinase (PI3K) and RalGDS. One of the best characterized effector molecules activated by Ras is Raf kinase. Activation of Raf initiates a phosphorylation cascade involving MEK and ERK protein kinases leading to the activation of transcription factors like Elk1.

Normally, Ras-signaling cascades are only transiently activated because GTPase's intrinsic hydrolyzing activity gradually converts GTP to GDP. This conversion is also enhanced by the presence of GAP proteins. However, there are mutant oncogenic Ras proteins that remain constitutively in the active GTP-bound form. Identified mutations are limited to a small number of sites that abolish GAP-induced hydrolysis of GTP, resulting in continuous stimulation of cellular proliferation. Oncogenic mutations in the ras gene are present in approximately 30% of all human cancers. Colon and pancreatic cancers have mutations in the K-ras gene, urinary tract and bladder cancers have mutations in the H-ras gene, and mutations in N-ras are associated with leukemia[4].

Kit Performance and Benefits

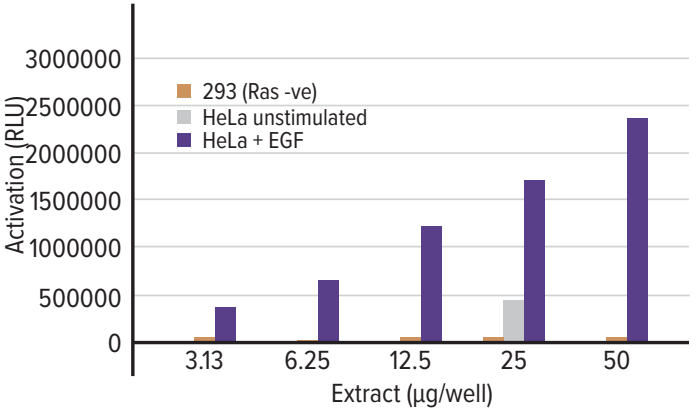
The Ras GTPase ELISA Kit is for research use only. Not for use in diagnostic procedures.

Detection limit: > 3 µg whole-cell extract/well or > 0.6 ng purified protein/well.

Range of Detection: The Ras GTPase Chemi ELISA can give relative quantitation of Ras-GTPase when used in the range of 3 to 25 µg of cell extract/well and compared to the provided control HeLa + EGF cell extract. (see graph below)

Cross-reactivity: Ras GTPase ELISA specifically detects activated H- and K-Ras in human and H-Ras in rodent samples.

Assay time: 4.5 hours.



Quantification of activated Ras: Increasing amounts of whole-cell extracts from unstimulated 293T/17 and EGF stimulated HeLa cells were assayed for Ras activity using the Ras GTPase Chemi ELISA Kit. To illustrate the Kit's specificity for activated Ras, 293T/17 cells which do not contain basal levels of activated Ras were used as a negative control. Data was also shown for unstimulated HeLa cells, which do contain basal levels of activated Ras. This data is provided for demonstration only.

Kit Components and Storage

Please store each component at the temperature indicated in the table below upon receipt of the kit. Kit components must be stored at the temperatures listed below for 24 hours prior to use.

Reagents	Quantity	Storage
Hela whole-cell extract (EGF treated, 2.5 µg/µl)	80 µl	-80°C
GST-Raf-RBD (2 mg/ml)	100 µl	-80°C
H-Ras antibody	11 µl	-20°C
Anti-rat HRP-conjugated IgG (0.25 µg/µl)	11 µl	-20°C
Protease Inhibitor Cocktail (PIC)	500 µl	-20°C
Lysis/Binding Buffer AM11	50 ml	4°C
10X Wash Buffer AM2	2 x 22 ml	4°C
10X Antibody Binding Buffer AM2	2.2 ml	4°C
Chemiluminescent Reagent	2 ml	4°C
Reaction Buffer	4 ml	4°C
96-well assay plate	1 ea	4°C
Plate sealer	2 ea	4°C

Additional materials required

- Multi-channel pipettor
- Multi-channel pipettor reservoirs
- Rocking platform at room temperature and 4°C
- Microplate luminometer or CCD camera-coupled imaging system
- PBS

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

Buffer Preparation and Recommendations

Preparation of Complete Lysis/Binding Buffer

We provide an excess of Lysis/Binding Buffer in order to perform the assay AND to prepare customized cell extracts. Prepare the amount of Complete Lysis/Binding Buffer required for the assay by adding 10 µl of Protease Inhibitor Cocktail per ml of Lysis/Binding Buffer (see the Quick Chart for Preparing Buffers in this section). Some of the protease inhibitors lose their activity after 24 hours once diluted. Therefore, we recommend using the Complete Lysis/Binding Buffer immediately for cell lysis. The remaining amount should be discarded if not used in the same day.

Preparation of 1X Wash Buffer

Prepare the amount of 1X Wash Buffer required for the assay as follows: For every 10 ml of 1X Wash Buffer required, dilute 1 ml 10X Wash Buffer AM2 with 9 ml distilled water (see the Quick Chart for Preparing Buffers in this section). Mix gently to avoid foaming. The 1X Wash Buffer may be stored at 4°C for one week. The Tween 20 contained in 10X Wash Buffer AM2 may form clumps, therefore homogenize the buffer by vortexing for 2 minutes prior to use.

Preparation of 1X Antibody Binding Buffer

Prepare the amount of 1X Antibody Binding Buffer required for the assay as follows: For every 10 ml of 1X Antibody Binding Buffer required, dilute 1 ml 10X Antibody Binding Buffer AM2 with 9 ml distilled water (see the Quick Chart for Preparing Buffers in this section)*. Mix gently to avoid foaming. Discard remaining 1X Antibody Binding Buffer after use. The BSA contained in the 10X Antibody Binding Buffer AM2 may form clumps, therefore homogenize the buffer by warming to room temperature and vortexing for 1 minute prior to use.

Diluted Primary Antibody

The primary Ras antibody recognizes H- and K-Ras in human and H-Ras in rodent samples. The supplied antibody will be diluted 1:500 in 1X Antibody Binding Buffer (see the Quick Chart for Preparing Buffers in this section). Avoid multiple freeze/thaw cycles.

Diluted HRP-conjugated Secondary Antibody

HRP-conjugated anti-rat IgG is used as the secondary antibody to detect bound primary antibody. The supplied antibody will be diluted 1:5000 in 1X Antibody Binding Buffer. This dilution should be made by performing a 1:10 dilution followed by a 1:500 dilution (see the Quick Chart for Preparing Buffers in this section). Avoid multiple freeze/thaw cycles.

Preparation of Chemiluminescent Working Solution

The Chemiluminescent Reagent and Reaction Buffer should be warmed to room temperature before use. These components are light sensitive, therefore, we recommend avoiding direct exposure to intense light during storage. Prior to use, place the Chemiluminescent Reagent and Reaction Buffer at room temperature for at least 1 hour. In a separate container, mix 1 volume of Chemiluminescent Reagent with 2 volumes of Reaction Buffer to prepare the Chemiluminescent Working Solution (see the Quick Chart for Preparing Buffers in this section). The Chemiluminescent Working Solution is stable for several hours. After the Chemiluminescent Working Solution is aliquoted into the wells, discard the remaining solution.

GST-Raf-RBD

The GST-Raf-RBD contains a Ras Binding Domain and is used to capture activated Ras on the glutathione-coated plate. GST-Raf-RBD must be aliquoted into small fractions to avoid freeze/thaws. Four tubes of the GST-Raf-RBD are provided and must be stored at -80°C upon receipt.

96-well assay plate

The 96-well assay plate is a solid plate. Determine the approprate number of wells required for testing. Cover the unused wells with a portion of the plate sealer while you perform the assay to prevent contamination. The content of the wells is stable at room temperature if kept dry and, therefore, can be used later for a separate assay. Return the plate to the foil pouch, seal and store at 4°C between uses.

Extract

The HeLa whole-cell extract (EGF treated) is provided as a positive control for Ras activation. Sufficient extract is supplied for 8 reactions per plate. This extract is optimized to give a strong signal when used at 25 µg/well. We recommend aliquoting the extract in 21 µl fractions and storing at -80ºC. Avoid multiple freeze/thaw cycles of the extract.

NOTE: The HeLa whole-cell extract (EGF treated) is sensitive to GTP hydrolysis at 4°C, thus we recommend thawing it no more than 15 minutes prior to use.

Quick Chart for Preparing Buffers**

Reagents to prepare	Components	For 1 well	For 1 strip (12 wells)	For 4 strips (48 wells)	For 12 strips (96 wells)
Complete Lysis/Binding Buffer	Protease inhibitor cocktail	0.55 µl	6.5 µl	26 µl	52 µl
	Lysis Buffer AM1	55 µl	643.5 µl	2.574 ml	5.148 ml
	TOTAL REQUIRED	**55.55 µl**	**650 µl**	**2.6 ml**	**5.2 ml**
1X Antibody Binding Buffer*	Distilled water	112.5 µl	1.26 ml	4.95 ml	9.9 ml
	10X Ab Binding Buffer AM2	12.5 µl	140 µl	550 µl	1.1 ml
	TOTAL REQUIRED	**125 µl**	**1.4 ml**	**5.5 ml**	**111 ml**
Primary Antibody 1/500 Dilution	Ras antibody	0.11 µl	1.3 µl	5.2 µl	10.4 µl
	1X Ab Binding Buffer	55 µl	648.7 µl	2.6 ml	5.2 ml
	TOTAL REQUIRED	**55.11 µl**	**650 µl**	**2.605 ml**	**5.210 ml**
Secondary Antibody Pre-Dilution (1/10)	HRP-conjugated antibody	1 µl	1 µl	1 µl	2 µl
	1X Ab Binding Buffer	9 µl	9 µl	9 µl	18 µl
	TOTAL REQUIRED	**10 µl**	**10 µl**	**10 µl**	**20 µl**
Secondary Antibody 1/500 Dilution	Pre-diluted HRP	0.11 µl	1.3 µl	5.2 µl	10.4 µl
	1X Ab Binding Buffer	55 µl	648.7 µl	2.6 ml	5.2 ml
	TOTAL REQUIRED	**55.11 µl**	**650 µl**	**2.605 ml**	**5.210 ml**
1X Wash Buffer	Distilled water	2.52 ml	30.6 ml	122.4 ml	243 ml
	10X Wash Buffer AM2	280 µl	3.4 ml	13.6 ml	27 ml
	TOTAL REQUIRED	**2.8 ml**	**34 ml**	**136 ml**	**270 ml**
Chemiluminescent Working Solution	Chemiluminescent Reagent	17 µl	217 µl	867 µl	1.734 ml
	Reaction Buffer	34 µl	434 µl	1.734 ml	3.468 ml
	TOTAL REQUIRED	**51 µl**	**651 µl**	**2.601 ml**	**5.202 ml**

* Volumes listed refer to the preparation of buffers for diluting both the primary & secondary antibodies.

** The Quick Chart includes an excess of components to perform the assay.

Preparation of Whole-Cell Extract

This procedure can be used for a confluent cell layer of 10 cm^2 (100 mm dish) or 2 x 10^7 cells.

1. Treat the cells as required for Ras activation.
2. Wash the cells with 5 ml ice-cold PBS (10 mM phosphate buffer, pH 7.5, 150 mM NaCl).
3. For adherent cells add 500 µl of Complete Lysis/Binding Buffer and scrape cells (with a rubber policeman). For suspension cells resuspend cell pellet in 1 ml Complete Lysis/Binding Buffer.
4. Transfer cells to a microcentrifuge tube. Incubate 15 minutes at 4°C.
7. Vortex tube for 10 seconds and then centrifuge for 10 minutes at 14,000 rpm at 4°C.
8. Collect the supernatant at 4°C.
9. Measure the protein content by a Bradford-based assay.
10. For best results, extracts should be used immediately in the Ras ELISA.

10X PBS	**For 250 ml, mix:**
0.1 M phosphate buffer, pH 7.5	3.55 g Na_2HPO_4 + 0.61 g KH_2PO_4
1.5 M NaCl	21.9 g
27 mM KCl	0.5 g

Adjust to 250 ml with distilled water. Prepare a 1X PBS solution by adding 10 ml 10X PBS to 90 ml distilled water. Sterilize the 1X PBS by filtering through a 0.2 µm filter. The 1X PBS is at pH 7.5. Store the filter-sterilized 1X PBS solution at 4°C.

OPTIONAL - GTPγS or GDP Treatment

The protocol below is provided as an optional procedure for the production of positive and negative controls for Ras activation. GTPγS acts as an activator while GDP acts as an inhibitor to Ras activation. Use > 200 µg of extract for each treatment.

1. Dilute the test extracts to desired concentration in Complete Lysis/Binding Buffer (>200 µg per well is recommended).
2. To each tube, add 0.5M EDTA pH 8.0 to a final concentration of 10 mM.
3. To each tube, add 10 mM GTPγS or 100 mM GDP to a final concentration of 0.1 mM and 1.0 mM, respectively.
4. Incubate at 30°C for 15 minutes.
5. To each tube, add 1M $MgCl_2$ to a final concentration of 60 mM.
6. Extracts should be used immediately in the Ras ELISA.

Ras GTPase Chemi ELISA

Determine the appropriate number of microwell strips required for testing samples, controls and blanks in duplicate. Cover the unused wells with a portion of the plate sealer while you perform

the assay. The content of these wells is stable at room temperature if kept dry and, therefore, can be used later for a separate assay. Return the plate to the foil pouch, seal and store at 4°C between uses.

Prepare the Complete Lysis/Binding Buffer, 1X Wash Buffer and 1X Antibody Binding Buffer as described in the section Buffer Preparation and Recommendations. Multi-channel pipettor reservoirs may be used for dispensing the Complete Lysis/Binding Buffer, Wash Buffer, Antibody Binding Buffer and Chemiluminescent Working Solution into the wells being used.

Step 1: Binding of Ras

IMPORTANT: For optimal kit performance, kit components must be stored at the recommended storage temperatures indicated on page 5 of the manual for 24 hours prior to use.

1. Add 2 µg of GST-Raf-RBD diluted in 50 µl of Complete Lysis/Binding Buffer to each well to be used. (1 µl of GST-Raf-RBD in 49 µl Complete Lysis/Binding Buffer per well).
2. Use the provided adhesive cover to seal the plate. Incubate for 1 hour at 4°C with mild agitation (100 rpm on a rocking platform).
3. Wash each well 3 times with 200 µl 1X Wash Buffer. For each wash, flick the plate over a sink to empty the wells, then tap the inverted plate 3 times on absorbent paper towels.
4. **Sample wells:** Dilute test extracts to desired concentration in Complete Lysis/Binding Buffer. Sample can be used at 50-200 µl per well, depending on stock concentration. We recommend using 10-100 µg of extract diluted in Complete Lysis/Binding Buffer per well.

   Positive control wells: Thaw the provided HeLa (EGF treated) extract on ice for no more than 15 minutes prior to use. Add 25 µg of this extract diluted in 50 µl of Complete Lysis/Binding Buffer per well (10 µl of extract in 40 µl of Complete Lysis/Binding Buffer per well).

   Blank wells: Add 50 µl Complete Lysis/Binding Buffer only per well.
5. Cover the plate and incubate for 1 hour at room temperature with mild agitation (100 rpm on a rocking platform).
6. Wash each well 3 times with 200 µl 1X Wash Buffer (as described in Step 1, No. 3)

Step 2: Binding of primary antibody

1. Add 50 µl diluted H-Ras antibody (1:500 dilution in 1X Antibody Binding Buffer) to wells.
2. Cover the plate and incubate for 1 hour at room temperature without agitation.
3. Wash the wells 3 times with 200 µl 1X Wash Buffer (as described in Step 1, No. 3).

Step 3: Binding of secondary antibody

1. Add 50 µl diluted HRP antibody (1:5000 dilution in 1X Antibody Binding Buffer) to all wells being used.
2. Cover the plate and incubate for 1 hour at room temperature without agitation.
3. During this incubation, place Chemiluminescent Reagent and Reaction Buffer at room temperature.
4. Wash the wells 4 times with 200 µl 1X Wash Buffer (as described in Step 1, No. 3).

Step 4: Chemiluminescent detection

1. Add 50 µl room-temperature Chemiluminescent Working Solution to all wells being used.
2. Read chemiluminescence using a luminometer or CCD camera system. Readings should be taken within 15 minutes to minimize changes in signal intensity.

References

1. Herrman C. *et al* (1996) *J. Biol. Chem.* 271(12): 6794-6800.
2. Alberts *et al* (1983) *Molecular Biology of the Cell, 3rd Edition*: 206-207.
3. Kontani K. *et al* (2002) *J. Biol. Chem.* 277(43): 41070-41078.
4. Adeji A. *et al* (2001) *J. National Cancer Institute* 93(14): 1062-1074.

Section A. Troubleshooting Guide

Problem/question	Possible cause	Recommendation
No signal or weak signal in all wells	Omission of key reagent	Check that all reagents have been added in all wells in the correct order.
	Substrate or conjugate is no longer active	Test conjugate and substrate for activity by mixing HRP and Chemiluminuescent Working Solution together.
	Enzyme inhibitor present	Sodium azide will inhibit the peroxidase reaction. Follow our recommendations to prepare buffers.
	Plate reader settings not optimal	Verify the measurement mode and filter settings on the plate reader.
	Incorrect assay temperature	Bring substrate to room temperature before using.
	Inadequate volume of Chemiluminescent Working Solution	Check to make sure that correct volume is delivered by pipette.
High background in all wells	Measurement time too long	Reduce integration time or exposure time on luminometer or CCD camera.
	Concentration of antibodies too high	Increase antibody dilutions.
	Inadequate washing	Ensure all wells are filled with Wash Buffer and follow washing recommendations .
High background in sample wells	Too much cellular extract per well	Decrease amount of cellular extract.
	Concentration of antibodies is too high	Perform antibody titration to determine optimal working concentration. Start by using 1:1,000 for primary antibody and 1:10,000 for secondary antibody. The sensitivity of the assay will decrease.
No signal or weak signal in sample wells	Not enough extract per well	Increase amount of extract. Do not exceed 500 µg/well.
	Ras is poorly activated or inactive.	Perform a time course for Ras activation in the studied cell line.
	Extracts are not from correct species	Refer to cross-reactivity information on page 4.

Technical Services

If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

Active Motif North America

Toll free: 877.222.9543
Direct: 760.431.1263
Fax: 760.431.1351
E-mail: tech_service@activemotif.com

Active Motif Europe

UK Free Phone: 0800/169 31 47
France Free Phone: 0800/90 99 79
Germany Free Phone: 0800/181 99 10
Direct: +32 (0)2 653 0001
Fax: +32 (0)2 653 0050
E-mail: eurotech@activemotif.com

Active Motif Japan

Direct: +81 (0)3 5225 3638
Fax: +81 (0)3 5261 8733
E-mail: japantech@activemotif.com

Active Motif China

Direct: (86)-21-20926090
Cell Phone: 18521362870
E-mail: techchina@activemotif.com

Visit Active Motif online at **activemotif.com**