Design and Use of a Mammalian Protein-Protein Interaction Trap (MAPPIT)

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Abstract

Identifying the interaction partners of a protein is a straightforward way to gain insight into the protein's function and to position it in an interaction network such as a signal transduction pathway. Various techniques have been developed to serve this purpose, and some are specifically designed to study posttranslational modifications in mammalian proteins and to clarify their normal physiological context. However, several intrinsic constraints limit the use of these technologies, and most are not suitable for screening for new interacting partners. In the Mammalian Protein-Protein Interaction Trap (MAPPIT) Protocol described here, knowledge of cytokine receptor signaling has been used to design a versatile genetic tool that can be used analytically and for detection of new protein-protein interactions in mammalian cells.

Introduction

Although it has been more than 12 years since the original method was published (*1*), the yeast two-hybrid assay and its derivatives are still very popular in the search for new protein-protein interactions. Through the years, numerous variations on the technology have been developed, including adaptations to perform the analysis in mammalian cells, which provides a more appropriate environment for studying interactions between mammalian proteins. Other genetic strategies designed specifically to dissect interactions among mammalian cells include the Ras recruitment system, the ubiquitin-based split-protein sensor method, and fluorescence resonance energy transfer (FRET)/bioluminescence resonance energy transfer (BRET)-based assays [reviewed in (*2*)]. Since very sensitive mass spectrometry methods have become available, biochemical approaches utilizing affinity-based purification of protein complexes are also being applied in the elucidation of protein interaction networks (*3*, *4*).

Here, we describe a protocol for a novel mammalian two-hybrid system that is based on insights in type I cytokine receptor signal transduction. Cytokine receptor clustering following ligand binding triggers the cross-activation of associated Janus kinase (JAK). These kinases phosphorylate specific tyrosine residues along the receptor tails, which then become the docking sites for signal transducer and activator of transcription (STAT) signaling molecules. Recruited STATs are subsequently activated by phosphorylation, and migrate to the nucleus where they induce specific target gene transcription (*5*) (Fig. 1A). The mammalian protein-protein interaction trap (MAPPIT) strategy takes advantage of the fact that activated JAK kinases are able to phosphorylate STAT recruitment sites in trans (*6*, *7*). The bait protein is fused to a mutant receptor chimera that still allows JAK activation, but from which STAT target sites have been eliminated. The prey protein is tethered to a receptor fragment containing functional STAT recruitment sites. Upon bait-prey interaction, the prey chimeras are phosphorylated, and the recruited STAT molecules are activated and induce transcription of a reporter gene under the control of a STAT-responsive promoter (Fig. 1B).

Compared to other mammalian two-hybrid methods, MAPPIT offers the combined advantages of a near-optimal physiological context, and separation of interactor and effector zones. In addition, because signals are ligand-dependent, and variant bait chimeras can be used that are stimulated by different ligands, rapid discrimination of false positives and preliminary mapping of the interaction site on the bait protein are possible (*6*, *7*).

Research in biology is more and more approached from a holistic point of view; this is exemplified in molecular biology by high-throughput technologies such as complete genome sequencing and cDNA microarray analysis. General strategies to identify protein interaction networks in mammalian cells, however, currently suffer from high numbers of false positives and limited sensitivity. To date, no truly robust methods exist that are compatible with the large-scale screening necessary to draw reliable interaction maps of complex processes or signaling pathways in a mammalian cell, let alone its complete interactome. To allow screening of cDNA libraries for previously unidentified interaction partners, a specific HEK293-derived cell line was developed with the following features. First, stable expression of the bait and of the prey cDNAs can be obtained by targeted recombination and by retroviral infection through the murine ecotropic receptor, respectively. Second, clones harboring interacting bait and prey can be selected through a stably integrated puromycin resistance marker, under control of the STAT-responsive *rPAP1* promoter. Also, a strategy has been developed to efficiently recover the integrated prey cDNA from candidate colonies. The feasibility of this concept has been demonstrated by the successful screening of complex libraries for proteins interacting with a motif containing the tyrosine at amino acid position 402 of the erythropoietin (Epo) receptor (EpoR) (*6*).

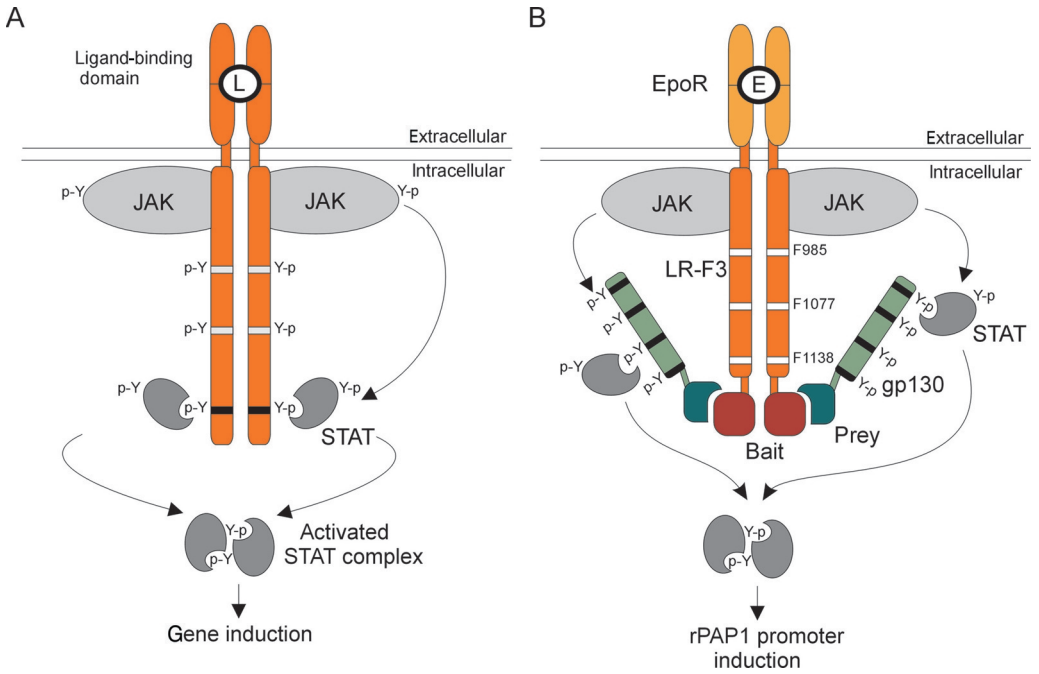

Fig. 1. (**A**) Schematic representation of the JAK-STAT pathway. Binding of a cytokine to its cognate receptor leads to clustering and rearrangement of the receptor chains, resulting in cross-activation of receptor-anchored JAK. In turn, these JAKs phosphorylate specific tyrosines within the intracellular receptor tails. These phosphotyrosines subsequently act as recruitment sites for various signaling molecules, including the STATs, which are latent transcription factors that migrate to the nucleus after phosphorylation and dimerization, where they induce specific STAT-responsive genes. L, ligand. (**B**) Principle of the MAPPIT system. A heterologous bait polypeptide is fused to a receptor variant lacking functional STAT3 recruitment sites. In the configuration shown, a receptor chimera consisting of the extracellular domain of the homodimeric EpoR fused to the transmembrane and cytosolic domains of the LR-F3 is used. Tyrosine to phenylalanine mutations eliminate the functional STAT3 recruitment site at position 1138 and also other recruitment sites at positions 985 and 1077 linking to the Ras pathway and to JAK-STAT signaling inhibitors. A heterologous prey polypeptide is fused to a receptor fragment of gp130 containing four functional STAT3 recruitment sites. If bait and prey interact, the recruited receptor fragment is phosphorylated upon ligand-induced receptor activation, and a STAT3-dependent signal is obtained. This process can be monitored using a STAT3 responsive reporter gene. E, Epo [Adapted and reprinted by permission from *Nature Cell Biology* (http://www.nature.com/ncb), vol. 3, no. 12, pp. 1114-1119, copyright (2001) Macmillan Publishers Ltd. (*6*)].

Materials

0.45 μm nitrocellulose filters

4-mm diameter paper discs (Whatman, 3MM Chr, #3030917)

96-well black tissue culture plates with clear bottom (Costar, #3603)

Note: If microscopic inspection is not required, standard tissue culture-grade, black well plates can also be used; they are less expensive.

150-cm^2 peel-off flask (TPP, Trasadingen, Switzerland, # 90551)

6-well plates for tissue culture

96-well plates for tissue culture

75-cm^2 flasks for tissue culture

70-μm cell strainer (Falcon, #352350)

Cell dissociation agent (Invitrogen, #13151-014)

Flp-In system (Invitrogen, #V6010-20)

GalactoStar kit β-galactosidase assay (Tropix, Bedford, MA, #BM100S)

GenoPrep streptavidin beads (GenoVision, #G210.002)

Human Epo (R&D Systems, #287-TC-500)

Hybond-C extra (Amersham Biosciences AB, #RPN203E)

Mouse leptin (R&D Systems, #498-OB-05M)

QIAshredder spin column (Qiagen, #79656)

OneStep reverse transcriptase-polymerase chain reaction (RT-PCR) kit (Qiagen, #210212)

Protein-G-sepharose (Amersham Biosciences AB, #17-0618-01)

SuperSignal chemiluminescent substrate (Pierce, #34080)

RNeasy kit (Qiagen, #74106)

Chemicals

Adenosine 5′ triphosphate (ATP) (Sigma-Aldrich, #A-2383)

β-mercaptoethanol

Coenzyme A (Sigma-Aldrich, #C-3019)

Complete proteinase inhibitor cocktail (Roche, #1697498)

Desoxycholate

Dithiothreitol (DTT)

D-luciferin (Duchefa, Haarlem, Netherlands, #L1349)

Dimethyl dicarbonate (DMPC) (Sigma-Aldrich #D-5520)

Dimethylsulfoxide (DMSO)

Dulbecco's modified Eagle's medium (DMEM)

Ethylenediaminetetraacetic acid

Fetal calf serum (FCS)

Gelatin

Glycerol

Hepes (Invitrogen, #15360-056)

Hygromycin B (Invitrogen, #10687-010)

$(MgCO_3)_4Mg(OH)_2 \bullet 5H_2O$

$MgSO_4$

NaF

Na_2HPO_4

Nonfat dry milk

Nonidet P-40 (NP-40) (Calbiochem-Novabiochem Corporation, La Jolla, CA, #492015)

Phosphate-buffered saline (PBS) with Ca^{2+} and Mg^{2+} (Invitrogen, #14040-083)

PBS without Ca^{2+} and Mg^{2+} (PBSA) (Invitrogen, #14190-084)

Polybrene (Sigma-Aldrich #H-9268)

Propidium iodide

Puromycin (Sigma-Aldrich #P-8833)

Sodium dodecyl sulfate (SDS)

Sodium vanadate

Trans-1,2-diaminocyclo-hexane-N,N,N′,N′-tetra acetic acid (CDTA) (Sigma-Aldrich #D-1383)

Tricine

Triton X-100

Trypsin-EDTA (Invitrogen, #25300-054)

Tween-20

Zeocin (Invitrogen, #R250-01)

Plasmids and DNA

Biotinylated gp130-specific oligonucleotide (5′-GGGCTGGGTAGACTCGGATCTTGAGAAGAC-3′-biotin)

Forward gp130-specific primer (5′-GGCATGGAGGCTGCGACTG-3′)

Reverse 3′ LTR-specific primer (5′-TCGTCGACCACTGTGCTGGC-3′)

Plasmid pSEL1 (*6*)

Plasmid pMG1 (*6*)

Plasmid pCEL1f (*6*)

Plasmid pBG1 (*6*)

Murine retroviral pBABE vector, containing the Moloney leukemia virus long terminal repeat (LTR) and Ψ sequences (available from G. Nolan, Department of Molecular Pharmacology, Stanford University, Stanford, CA 94305, USA, gnolan@stanford.edu)

pXP2d2-rPAP1-luci (a STAT-dependent reporter gene) (pXP2d2 available from S. Nordeen, Colorado Health Sciences Center, Department of Pathology, Denver, CO 80262, USA, steve.nordeen@uchsc.edu)

pUT651 (a constitutively active reporter gene that produces β-galactosidase; Eurogentec, Seraign, Belgium)

Cell Lines

Human embryonic kidney (HEK) 293T (ATCC)

HEK293-16 (*6*)

Plat-E cells (available from T. Kitamura, Division of Hematopoietic Factors, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan, kitamura@ims.u-tokyo.ac.jp)

Antibodies

Polyclonal goat antibody to human EpoR (R&D Systems, #AF-322-PB)

Alexa-conjugated donkey antibody to goat (Molecular Probes, #A-11055)

Anti-PY-20-biot, biotin-conjugated antibody against phosphotyrosine (Transduction Labs, #P11123)

Streptavidin-horseradish peroxidase (HRP) (Jackson ImmunoResearch laboratories, Inc., West Grove, PA 19390, #016-030-084)

Equipment

Chemiluminescence meter (for example, Topcount, PerkinElmer Life Sciences)

Fluorescence-activated cell sorter (FACS) (for example, FACSCalibur, Becton Dickinson)

Magnetic particle concentrator (MPC) for use with 1.5-ml tubes (for example, Dynal MPC, Dynal Biotech #120.04)

Standard tissue culture equipment (laminar flow hood, CO_2 incubator, and so forth)

Thermal cycler

Recipes

Recipe 1: Growth Medium

DMEM supplemented with 10% FCS (v/v).

Recipe 2: 2.5 M $CaCl_2$

Prepare 10 ml in dH_2O. Filter sterilize by passage through a 0.45-μM nitrocellulose membrane. Store at –20°C.

Recipe 3: 2× Hepes-Buffered Saline (HeBS)

NaCl	280 mM
Na_2HPO_4	1.5 mM
Hepes	50 mM

Dissolve in 90 ml of dH_2O. Adjust pH to 7.05 with NaOH and adjust the volume to 100 ml. Filter sterilize by passage through a 0.45-μM nitrocellulose membrane. Store at –20°C.

Recipe 4: Epo Stock Solution

Dilute human Epo in Serum-Free Medium (Recipe 8) to a final concentration of 5 μg/ml, aliquot in 500 μl volumes, and store at –20°C.

Recipe 5: Luciferase Lysis Buffer (LLB)

Reagent	*Final Concentration*
Tris-phosphate pH 7.8	25 mM
DTT	2 mM
CDTA	2 mM
Glycerol	10%
Triton X-100	1%
Prepare	50 ml in dH_2O.

Recipe 6: Luciferase Substrate Buffer (LSB)

Reagent	*Final Concentration*
Tricine	20 mM
$(MgCO_3)_4Mg(OH)_2 \bullet 5H_2O$	1.07 mM
$MgSO_4$	2.67 mM
DTT	33.3 mM
EDTA	0.1 mM
Coenzyme A	270 μM
ATP	530 μM
D-luciferin	470 μM
Prepare	50 ml in dH_2O.

Recipe 7: FACS Buffer

Reagent	*Final Concentration*
FCS	1%
EDTA	0.5 mM

Prepare in PBSA for a final volume of 500 ml.

Recipe 8: Serum-Free Medium

Add Hepes (pH 7.5) to a final concentration of 10 mM to DMEM.

Recipe 9: Modified RIPA Buffer

Reagent	*Final Concentration*
NaCl	200 mM
Tris-HCl pH 8.0	50 mM
NP-40	1%
Desoxycholate	0.5%
SDS	0.05%
EDTA	2 mM
Sodium vanadate	1 mM
NaF	1 mM
β-glycerophosphate	20 mM

Prepare 50 ml in dH_2O. Add one tablet of complete proteinase inhibitor cocktail just before use.

Recipe 10: PBS-T

Prepare a 0.1% Tween-20 (v/v) solution in PBS for a final volume of 1000 ml.

Recipe 11: Cell-Freezing Medium

20% FCS (v/v) and 10% DMSO (v/v) in DMEM. Prepare 1 ml for 1×10^6 to 5×10^6 cells.

Recipe 12: Solution A

NaOH	0.1 M
NaCl	0.05 M

Prepare in dH_2O for a final volume of 50 ml. Add DMPC to a final concentration of 0.1%, shake vigorously, incubate 1 hour at room temperature, and autoclave.

Recipe 13: Solution B

NaCl	0.1 M

Prepare in dH_2O for a final volume of 50 ml. Add DMPC to a final concentration of 0.1%, shake vigorously, incubate 1 hour at room temperature, and autoclave.

Recipe 14: RNase-Free Distilled Water

To 1000 ml of dH_2O, add DMPC to a final concentration of 0.1%, shake vigorously, incubate 1 hour at room temperature and autoclave.

Recipe 15: Binding Buffer

Reagent	*Final Concentration*
NaCl	1 M
Tris-HCl pH 7.5	10 mM
EDTA	1 mM
Tween-20	0.01%

Prepare in RNase-Free Distilled Water (Recipe 14) for a final volume of 50 ml.

Recipe 16: Washing Buffer

Reagent	*Final Concentration*
NaCl	0.15 M
Tris-HCl pH 7.5	10 mM
EDTA	1 mM
Tween-20	0.01%

Prepare in RNase-Free Distilled Water (Recipe 14) for a final volume of 50 ml.

Instructions

Vectors for the Protein Chimeras

Four plasmid vectors were developed for use in the MAPPIT system. pSEL1 and pMG1 are designed for analytical applications of the method, and pCEL1f and pBG1 are for screening. The structure of the vectors and the protein chimeras they encode are shown in Figure 2. Subcloning is performed using standard methods. We describe briefly the optimal restriction enzymes to use for cloning and creation of the chimeric fusion proteins.

The pSEL1 plasmid is derived from pSV-SPORT, which carries an early SV40 promoter for low-level expression in mammalian cells (for example, in HEK293T cells). This vector encodes a chimeric receptor consisting of the extracellular part of the EpoR fused to the transmembrane and intracellular parts of the leptin receptor (LR) F3 variant that lacks phosphorylatable tyrosine residues (LR-F3). Ideally, cloning of the bait protein (or a portion thereof) is done using SacI and NotI restriction sites, because such fragments can also be inserted into the pCEL1f vector if desired.

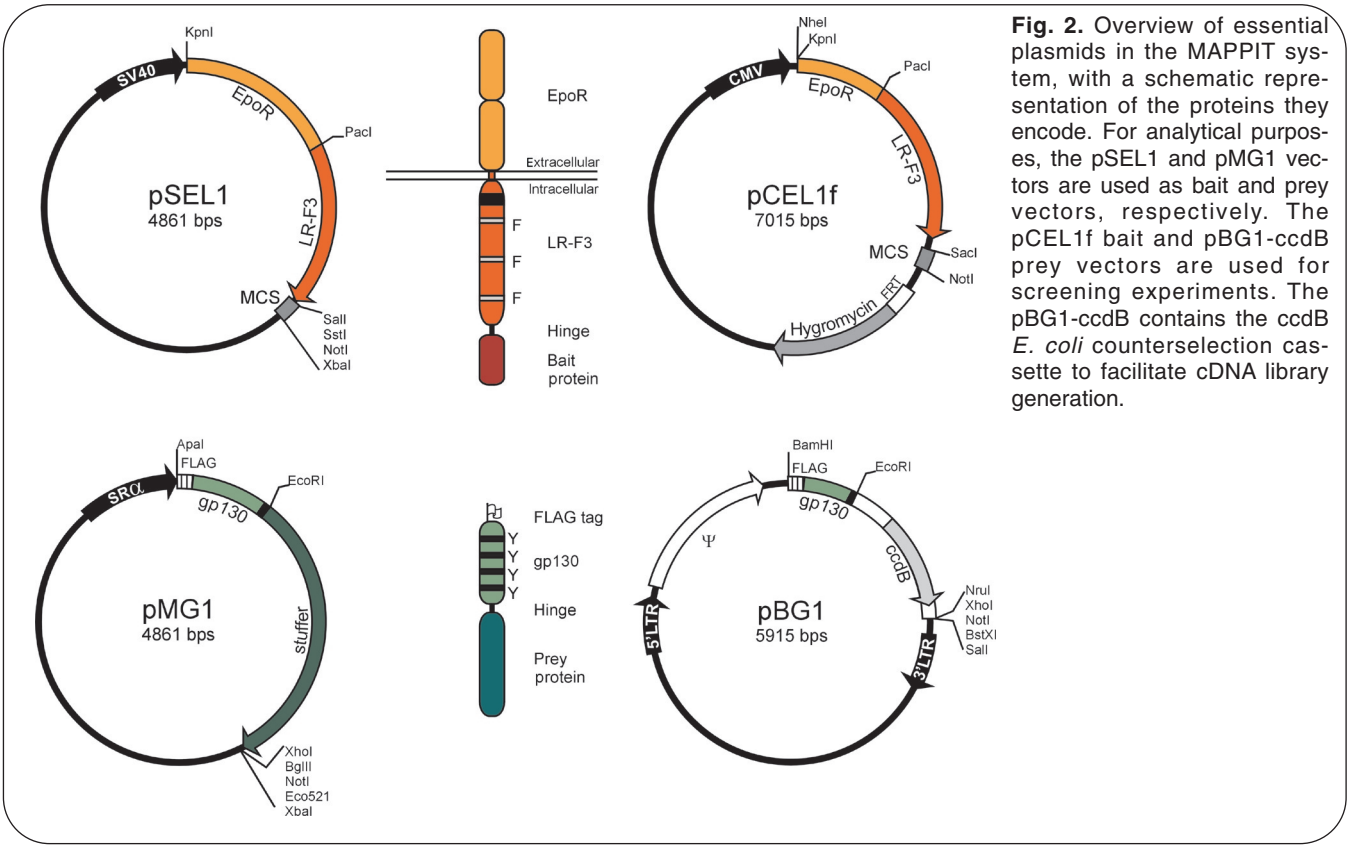

Fig. 2. Overview of essential plasmids in the MAPPIT system, with a schematic representation of the proteins they encode. For analytical purposes, the pSEL1 and pMG1 vectors are used as bait and prey vectors, respectively. The pCEL1f bait and pBG1-ccdB prey vectors are used for screening experiments. The pBG1-ccdB contains the ccdB *E. coli* counterselection cassette to facilitate cDNA library generation.

The protein prey of interest (or a portion thereof) is cloned COOH-terminal to the gp130 fragment. The pMG1 plasmid is derived from the mammalian expression vector pMET7, which contains the strong *SRα* promoter (*8*). This vector encodes the FLAG-tagged gp130 fragment with a glycine-glycine-serine (GGS) amino acid linker region that precedes the multiple cloning site. Ideally, EcoRI and NotI restriction sites are used, because such fragments can also be cloned in the retroviral pBG1 vector if desired.

The pCEL1f vector is based on the pcDNA5/FRT plasmid (Flp-In system). The vector contains the cytomegalovirus (CMV) promoter and a Flp recombination target (FRT) site, followed by a hygromycin resistance cassette to permit Flp recombinase-mediated integration and subsequent selection. The coding sequence for the chimeric receptor is inserted into this plasmid. Bait proteins can be cloned after the COOH-terminal hinge sequence using SacI and NotI restriction sites.

cDNA libraries are generated in the murine retroviral pBABE vector containing the Moloney leukemia virus long terminal repeat (LTR) and Ψ sequences. Prey cDNA are inserted between EcoRI and NotI or EcoRI and XhoI restriction sites at the 3′ end of the gp130 sequence. This removes a stuffer sequence encoding the bacterial "control of cell death B" (ccdB) protein to permit counter selection for self-ligated plasmids.

Analytical Applications of MAPPIT

We describe the assay using HEK293T cells. Other cell lines can be used; however, the *rPAP1* promoter is also sensitive to STAT5, so they must not express high levels of endogeneous STAT5, in order to prevent false positive results. HEK293T cells are cultured in Growth Medium (Recipe 1) in a humidified atmosphere at 37°C and 8 to 10% CO_2. Cells should be kept subconfluent.

Briefly, the cells are transfected with the bait plasmid, the prey plasmid, and a STAT-dependent reporter gene (pXP2d2-rPAP1-luci). The stimulation of the reporter gene is used as a measure of the interaction between the bait and prey proteins. The cells are also transfected with a constitutively expressed reporter gene (pUT651 encoding β-galactosidase) to normalize transfection variations. Sufficient cells should be plated to allow the experiments to be performed in triplicate. Not only can the reporter gene expression be assayed to quantify protein interactions, but expression of the bait and prey proteins can be verified by Western blot analysis.

Transfection

1. For each transfection condition, plate between 2.5×10^5 and 4×10^5 subconfluent HEK293T cells in a 10-cm^2 well with 2 ml Growth Medium (Recipe 1).
2. Grow overnight at 37°C, 8 to 10% CO_2.
3. 1 to 4 hours before transfection, replace medium with 1.8 ml of fresh Growth Medium (Recipe 1) per well.
4. For each well, prepare a DNA mixture containing 1 μg of pSEL1 bait construct, 1 μg of pMG1 prey construct, 200 ng of pXP2d2-rPAP1-luci, and 25 ng of pUT651. Adjust with dH_2O to a final volume of 135 μl, and add 15 μl of 2.5 M $CaCl_2$ (Recipe 2) to the DNA mixture.

   Note: Different ratios of bait and prey plasmids may yield better results in some settings.
5. Gently drop 125 μl of this DNA-$CaCl_2$ mixture into 125 μl of 2× HeBS (Recipe 3) in a 1.5-ml tube, while vortexing to mix.
6. After vortexing the mixture for 5 s more, leave it to precipitate for at least 20 min at room temperature.
7. Vortex vigorously for 5 s.
8. Add 200 μl of the precipitation mixture to each well of cells.

   Note: The precipitate can be checked microscopically; it should be small (almost invisible at a 100× magnification) to obtain optimal transfection efficiencies.
9. Leave precipitate on cells overnight in the incubator at 37°C, 8 to 10% CO_2.
10. Remove the DNA-containing medium and wash the cells once with 1 ml of PBS.
11. Add fresh Growth Medium (Recipe 1).

Cell transfer and stimulation

1. Remove the Growth Medium and add 200 μl of cell dissociation agent to each well.
2. Gently tap plate to detach all cells.
3. Add 1.8 to 2.3 ml (depending on confluency status) of Growth Medium (Recipe 1) and triturate using a 1-ml pipette to break cell clusters.
4. Plate 50 μl of cell suspension per well of a black-well plate, in triplicate for every stimulation condition.

   Note: We recommend three wells for unstimulated cells, three wells for erythropoietin (Epo)-stimulated cells, and three wells in a separate plate for measurement of β-galactosidase activity, which is used to normalize transfection variations. The remainder of the cells can be used for Western blot analysis to verify bait and prey expression.
5. Add 50 μl of Growth Medium (Recipe 1) with or without Epo Stock Solution (Recipe 4) diluted and added to deliver 10 ng/ml of Epo.

   Note: Final concentration of Epo should be 5 ng/ml.
6. Grow the cells in the presence of Epo for 24 hours.

Reporter gene assays

β-galactosidase activity is measured as a control for transfection variability. We usually use the GalactoStar kit from Tropix and perform the assay as described by the manufacturer. We describe the STAT-dependent luciferase reporter assay in more detail.

1. Remove the medium from the Epo-stimulated and unstimulated cells.
2. Lyse the cells for 10 min in 50 μl of LLB (Recipe 5).
3. Add 35 μl of LSB (Recipe 6).
4. Measure the light produced instantly by putting the plate in a luminescence counter.

MAPPIT Screening Procedure

For screening constructs, we use a HEK293-derived cell line (HEK293-16) with three distinct properties. First, the cells contain an integrated FRT cassette (Flp-In), allowing rapid and efficient integration of chimeric bait proteins through targeted recombinational insertion. Selection creates an isogenic cell pool that consists of a population with homogeneous bait expression level.

Second, HEK293-16 cells contain the murine ecotropic EcoR receptor, which is essential for infection with Moloney murine leukemia virus-derived vectors. This system allows stable expression of complex prey cDNA libraries cloned in ecotropic retroviral vectors and prevents health hazards associated with the use of amphotropic vectors. The third property is the puromycin resistance cassette under control of the STAT3-dependent rPAP1-promoter that permits selection for bait-prey interactions.

The procedure involves subcloning the bait construct into the pCEL1f vector, transfecting the construct, and selecting isogenic pools of cells that express the bait. We describe a FACS method to confirm expression of the bait. Possible phosphorylation of the bait by activated JAKs can be monitored by Western analyses using antibodies to phosphotyrosine. The bait-expressing cells are then infected with a retroviral prey library, and the cells with interacting bait and prey are selected by puromycin resistance (Fig. 3)

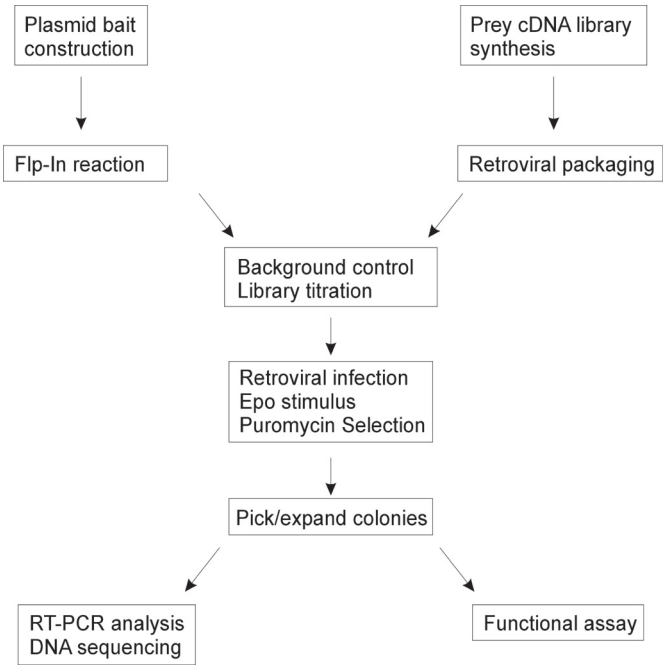

Fig. 3. Flow chart of the MAPPIT screening procedure. A typical screening experiment, without bait and retroviral prey library construction, takes 2 to 3 months. Two weeks are required to obtain sufficient bait-expressing cells using the Flp-In reaction. Background assays take about 3 weeks to visualize possible colonies. Determination of the retroviral titer can meanwhile be performed on the bait-expressing cells. Colony formation in the screening experiment takes 3 weeks, and cell expansion for RT-PCR and functional analysis requires another 2 to 3 weeks.

Preparation of isogenic cell pools stably expressing the bait

1. Subclone the bait constructs into the pCEL1f vector using standard molecular biology methods.
2. Follow the manufacturer's directions for the Flp-In recombinase reaction to generate isogenic cell pools stably expressing the bait.
3. Plate 4×10^6 to 5×10^6 HEK293-16 cells in a 75-cm^2 flask in 20 ml Growth Medium (Recipe 1).

   Note: HEK293-16 cells should be grown under selective conditions (that is, in the presence of 100 μg/ml zeocin) or should be kept at a low passage number.
4. Grow the cells overnight at 37°C, 8 to 10% CO_2.
5. Prepare a DNA mixture containing the bait plasmid (2 μg) and pOG44 plasmid (18 μg, Flp recombinase). Adjust with dH_2O to a final volume of 450 μl, and add 50 μl of 2.5 M $CaCl_2$ (Recipe 2).
6. Gently drop this DNA-$CaCl_2$ mixture into 500 μl 2 × HeBS (Recipe 3) while vortexing.
7. After vortexing for 5 s more, leave the solution to precipitate for at least 20 min at room temperature.
8. Vortex vigoruously for 5 s.
9. Add the complete precipitation mixture to the flask.
10. Incubate the cells overnight at 37°C, 8 to 10% CO_2.
11. Remove the DNA mixture and wash the cells once with 3 ml of PBS.

12. Add 20 ml of fresh Growth Medium (Recipe 1).
13. Grow the cells overnight at 37°C, 8 to 10% CO_2.
14. Detach the cells with 3 ml of Trypsin-EDTA.
15. Re-plate the cells in a 175-cm^2 tissue culture flask in 40 ml of Growth Medium (Recipe 1) at 15 to 20% confluency.

    Note: Do not seed cells at higher density because hygromycin-selection is critically dependent on cell growth, and the onset of selection and growth retardation in media supplemented with hygromycin B may take several days.
16. Grow the cells overnight at 37°C, 8 to 10% CO_2.
17. Add 100 µg/ml of hygromycin B to the Growth Medium for selection.
18. Grow the cells in hygromycin B medium for 5 days, then replace the medium with fresh Growth Medium (Recipe 1) containing 100 µg/ml of hygromycin B.
19. When colonies are clearly visible and no cells remain between the colonies, detach the cells with Trypsin-EDTA and plate them into fresh Growth Medium (Recipe 1) without hygromycin B.

    Note: An aliquot of the cells should be frozen immediately after the selection procedure to maintain a low passage number.

Confirming bait expression using FACS analysis

1. Plate 4×10^5 cells from the isogenic cell pools and from untransfected control HEK293-16 cells in 10-cm^2 tissue culture plates with 2 ml of Growth Medium (Recipe 1).
2. Grow the cells overnight at 37°C, 8 to 10% CO_2.
3. Detach and dissociate the cells with 500 µl of cell dissociation agent.
4. Add 500 µl FACS Buffer (Recipe 7).
5. Pellet the cells by centrifugation (2 min at 150*g*).
6. Resuspend the cells in 100 µl of FACS Buffer (Recipe 7) supplemented with polyclonal goat antibody to human EpoR at 2 µg/ml.
7. Incubate cells for 2 hours at 4°C with end-over-end rotation.
8. Add 600 µl of FACS Buffer (Recipe 7) and centrifuge for 2 min at 150*g*.
9. Wash the cells once more with 600 µl of FACS Buffer (Recipe 7), vortex gently, and centrifuge for 2 min at 150*g*.
10. Resuspend the cells in 200 µl of FACS Buffer (Recipe 7) supplemented with alexa-conjugated donkey antibody to goat at 4 µg/ml.
11. Incubate cells 45 min at 4°C with end-over-end rotation.
12. Add 600 µl of FACS Buffer (Recipe 7) and centrifuge for 2 min at 150*g*.
13. Resuspend the cells in 300 µl of FACS Buffer (Recipe 7) supplemented with 3 µM propidium iodide.

    Note: Propidium iodide is included to discriminate dead cells.
14. Perform flow cytometry data acquisition to analyze the expression of the bait protein.

    Note: The intensity of the signal obtained by FACS may vary among baits. Western blot analysis can be used as an alternative.

Verification of possible bait tyrosine phosphorylation by Western blot analysis

Some bait polypeptides may act as substrates for JAK activity and may thus become phosphorylated after receptor activation. Because no tyrosine phosphorylation sites are present in the intracellular part of the leptin receptor, tyrosine phosphorylation of the bait can be monitored by immunoprecipitation followed by Western blot analysis using antibodies to phosphotyrosine.

1. Grow 3×10^6 to 4×10^6 isogenic bait-expressing HEK293-16 cells for several days in 75-cm^2 flasks or in 9-cm plates to near-confluence in 20 ml Growth Medium (Recipe 1) at 37°C, 8 to 10% CO_2.
2. Replace Growth Medium with 20 ml Serum-Free Medium (Recipe 8) at least 30 min before stimulation.
3. Stimulate the cells under serum-free conditions for 5 to 10 min in the incubator with 5 ml of Epo Stock Solution (Recipe 4) that delivers 25 ng/ml Epo.

    Note: A set of unstimulated cells serves as a background control.
4. Remove the Epo-containing medium and wash the cells once with 5 ml of ice-cold PBSA.

5. Add 1 ml of ice-cold Modified RIPA Buffer (Recipe 9).
6. Incubate for 5 min on ice.
7. Collect the cell lysate by scraping, and transfer it into a 1.5-ml tube.
8. Centrifuge at 10,000*g* for 5 min at 4°C to remove the insoluble fraction.
9. Transfer the supernatant to a new 1.5-ml tube.
10. Add 25 µl of Protein-G-Sepharose to pre-clear the supernatant and incubate for 1 to 2 hours at 4°C with end-over-end rotation.
11. Centrifuge at 3500*g* for 3 min at 4°C.
12. Transfer the cleared supernatant to a new tube.
13. Add goat antibody to human EpoR to a final concentration of 2 µg/ml, and incubate overnight at 4°C with end-over-end rotation.
14. Add 25 µl of Protein-G-sepharose and incubate for another 2 hours at 4°C with end-over-end rotation.
15. Centrifuge at 3500*g* for 3 min at 4°C.
16. Carefully remove all supernatant and discard.
17. Wash the sepharose beads once with 1 ml of ice-cold Modified RIPA Buffer (Recipe 9).
18. Centrifuge at 3500*g* for 3 min at 4°C.
19. Carefully remove all remaining wash buffer.
20. Resuspend in 50 µl of Laemmli loading buffer with 5% β-mercaptoethanol for SDS-polyacrylamide gel electrophoresis (SDS-PAGE).
21. Heat to 95°C for 3 min.
22. Centrifuge at 10,000*g* for 5 min.
23. Store supernatant at –20°C or use directly for SDS-PAGE.
24. Load and electrophorese the samples on a 7.5% SDS-PAGE gel.
25. Electroblot onto a Hybond-C extra membrane.
26. Block the membrane in PBS-T (Recipe 10) supplemented with 1% gelatin for at least 1 hour.
27. Wash the membrane once with PBS-T (Recipe 10).
28. Incubate the membrane overnight with 1 µg/ml anti PY-20 antibody in PBS-T containing 0.1% gelatin.
29. Wash the membrane four times for 5 min each wash with PBS-T (Recipe 10).
30. Incubate the membrane with 0.1 µg/ml Streptavidin-HRP in PBS-T (Recipe 10) containing 5% nonfat dry milk.
31. Wash the membrane 4 times for 5 min each wash with PBS-T (Recipe 10).
32. Incubate the membrane with a chemiluminescent substrate and expose to autoradiography film.

Determining screening conditions

Background is determined by the number of colonies obtained after 3 weeks in the presence of Epo and puromycin, but without retroviral infection of a prey cDNA library. Background can consist of normal-sized colonies, more than 1 or 2 per flask, or of numerous tiny or small colonies, or of a mixture of both types. The background can depend on the bait used, the concentration of puromycin, the density of cells when plated, and the concentration of Epo.

Obviously, background screening conditions should approximate the screening conditions to be used to identify novel protein interactions. We typically use 3×10^6 cells per 150-cm^2 peel-off flask, 5 ng/ml Epo, and 1 µg/ml puromycin. However, we recommend testing a range of puromycin concentrations to establish the optimal concentration. Also, possible colonies arising from Epo-independent activation of the STAT-dependent rPAP promoter should be evaluated in cells treated with puromcyin and without Epo stimulation.

1. Plate 3×10^6 HEK293-16 cells expressing the chimeric bait protein per 150-cm^2 peel-off flask in Growth Medium (Recipe 1).

   Note: Sufficient cells should be plated to allow samples at each puromycin concentration with and without Epo stimulation. Thus, at least six flasks are required.

2. Grow the cells for 5 hours at 37°C, 8 to 10% CO_2.
3. Add Epo Stock Solution (Recipe 4) to a final concentration of 5 ng/ml Epo to half of the flasks.
4. Grow the cells for 24 hours at 37°C, 8 to 10% CO_2.
5. Add puromycin at concentrations of 1 μg/ml, 1.5 μg/ml, and 2 μg/ml to separate flasks.
6. Replace the medium once a week, maintaining the Epo and puromycin concentrations.
7. Examine the flasks for colony formation after 3 weeks by holding against a light source, or by microscopic analysis.

cDNA library construction and virus titer determination

Both oligo(dT)- and random-primed cDNA libraries are made using standard techniques. cDNAs are flanked by EcoRI and NotI restriction sites, allowing unidirectional cloning in pBG-type vectors. We have used a combination of both libraries for screening. Typically, at least 10^6 independent cDNA clones are obtained per library, starting from 5 μg of poly(A)$^+$ RNA.

Virus particles are produced by transfection of Plat-E cells (*9*) using standard methods. The virus titer is determined by cotransfection of one of the library-transfected flasks with a LacZ or enhanced green fluorescent protein (EGFP)-encoding retroviral vector. Subsequently, target cells are infected with serial dilutions of the virus particles obtained from the reporter- and library-transfected cells, and reporter expression in the target cells is measured.

1. Prepare a cDNA library by standard methods and subclone into the pBG1 vector.
2. Transfect Plat-E cells with the library in the pBG1 vector using standard methods.
3. Transfect one flask simultaneously with the cDNA library and a LacZ or EGFP retroviral-encoded reporter.
4. Infect target cells (such as HEK293-16 cells) with serial dilutions (1 in 3) from the reporter- and library-transfected cells.
5. Calculate viral titer by analyzing the LacZ (by β-galactosidase staining) or EGFP (by FACS analysis) expression in the target cells.

   Note: Viral titer should be calculated from dilutions that do not produce maximal infection.

Retroviral infection, stimulation, and selection

Values are given for a screen at standard conditions. Actual concentrations may be dictated by the results of the background evaluation experiment.

1. Dissociate bait-expressing HEK293-16 cells with Trypsin-EDTA.
2. Pass the cell suspension through a 70-μm cell strainer.
3. Plate 3×10^6 cells per 150-cm^2 peel-off flask in 20 ml Growth Medium (Recipe 1).

   Note: It is important to keep the cells subconfluent during propagation before plating.
4. Grow the cells for 5 hours at 37°C, 8 to 10% CO_2.
5. Add polybrene at 2.5 μg/ml final concentration to the cells.
6. Add an appropriate dilution of the virus and Epo Stock Solution (Recipe 4) (5 ng/ml final concentration). Virus and Epo can be added simultaneously in a small volume.

   Note: The appropriate virus dilution should produce an average integration of one retroviral prey cDNA per cell.
7. Grow the cells for 24 hours at 37°C, 8 to 10% CO_2.
8. Replace the virus-containing medium with 25 ml of Growth Medium (Recipe 1) containing 5 ng/ml Epo from Epo Stock Solution (Recipe 4), and 1 μg/ml puromycin.
9. Replace the medium once a week, maintaining the Epo and puromycin concentrations.
10. Inspect the flasks regularly (every day) after 2 weeks under selection conditions, and mark apparent colonies with a marking pen on the bottom of the flasks.

Colony selection, clone maintenance, and storage

1. Peel off the removable plastic cover and aspirate the medium.
2. Using sterile forceps, cover the colonies with 4-mm diameter paper discs that have been soaked in Trypsin-EDTA.
3. Incubate at room temperature for 3 to 5 min to allow colonies to detach from the plate.

4. Using sterile forceps, lift the colonies from the plate with the paper disc and transfer to a well of a 96-well plate containing 200 µl of Growth Medium (Recipe 1).
5. Shake the discs in the medium to release cells from the paper.
6. Grow the cells in the 96-well plates for 5 to 7 days, then remove the paper disc from the well.
7. Grow the cells to near-confluence (80 to 95%) before expanding. Growth Medium (Recipe 1) should be replaced every 5 days.
8. Dissociate the cells with 25 µl of Trypsin-EDTA, add 150 µl of Growth Medium (Recipe 1), resuspend, and transfer into new wells as follows.

   (a) Place 25 µl in one well (96-well plate) containing 150 µl of Growth Medium (Recipe 1) to be expanded for storage in liquid nitrogen.

   (b) Place 60 µl in another well (96-well plate) that contains 150 µl of Growth Medium (Recipe 1) for RT-PCR analysis (see below).

   (c)Place 60 µl in a 1-cm^2 well (48-well plate) containing 500 µl of Growth Medium (Recipe 1) for the functional assay (see below).
9. Add 200 µl of fresh Growth Medium (Recipe 1) to the original well as a back-up.
10. To the cells for storage (step 8a above), expand the cells into a 1-cm^2 well (48 well plate) and then into 25 cm^2 tissue culture flasks.
11. Freeze at least two vials for each clone in Cell-Freezing Medium (Recipe 11) and store in liquid nitrogen.

Functional assay for interacting proteins

1. Grow the cells in the 48-well plates (step 8c in "Colony selection, clone maintenance, and storage") until 70 to 90% confluent.
2. Remove the medium.
3. Add 40 µl of Trypsin-EDTA and wait 3 to 5 min to allow cells to detach.
4. Add 800 µl of Growth Medium (Recipe 1) and resuspend the cells.
5. Transfer 100 µl into each of 8 wells of a 96-well black tissue culture plate with clear bottom.
6. Grow the cells overnight at 37°C, 8 to 10% CO_2.

   Note: Any remaining cells in the 48-well plate can be recovered if necessary simply by adding 0.5 ml of Growth Medium (Recipe 1) and returning the cells to the incubator.
7. Perform calcium-phosphate transfection of the cells in the 96-well plate, as follows.

   Wells 1 and 2: Transfect with 50 ng of reporter vector (rPAP1-luciferase).

   Wells 3 and 4: Transfect with 50 ng of the LR-extracellular-LR-F3-bait construct and 50 ng of reporter vector.

   Wells 5 and 6: Transfect with 50 ng of the LR-extracellular-LR-F3 construct and 50 ng of reporter vector to test if the prey interaction is bait-dependent.

   Wells 7 and 8: Transfect with 50 ng of a wild-type LR construct to test for transfection efficiency and to serve as a stimulation control.

   Note: Cells obtained from the library screening stably express the EpoR-LR-F3-bait construct, by introducing the LR-extracellular-LR-F3-bait variant and by stimulation with leptin instead of Epo, we can discriminate between the stably and the transiently expressed bait construct. This way the bait-prey interaction is double-checked and made Epo-independent.
8. Allow the cells to grow for 48 hours after transfection.
9. Aspirate the medium and add 100 µl Growth Medium (Recipe 1) to wells 1, 3, 5, and 7 (which function as negative control), and add 100 µl Growth Medium supplemented with ligand [5 ng/ml human Epo (from Epo Stock Solution, Recipe 4) for well 2, or 100 ng/ml mouse leptin for wells 4, 6, and 8].
10. Grow the cells overnight at 37°C, 8 to 10% CO_2.
11. Measure luciferase activity as described above in Reporter Gene Assays.

Prey Recovery Using Streptavidin-Coated Magnetobeads

The protocol described below has been developed to allow fast and automated processing of candidate colonies starting from a low amount of input material. However, prey-specific sequences can be amplified equally well using more common methods, such as two-step RT-PCR and genomic PCR.

Prey-encoding mRNAs can be specifically purified on the basis of the presence of gp130-specific sequences at the 5′ end. Prey-specific RT-PCR can subsequently be performed using primers specific for the flanking gp130 and retroviral 3′ LTR sequences.

Because this procedure aims to amplify a specific and low abundance target from a complex background, one should take extreme care in order to prevent contamination that could lead to false positive bands. Therefore, all manipulations should be carried out in a laminar flow cabinet, and all disposables and reagents should be free from template contamination. Also, take the necessary measures to prevent RNase contamination of the samples. That is, wear gloves and use RNase-free disposables and reagents.

Preparation of GenoPrep streptavidin beads

1. Resuspend GenoPrep streptavidin beads by gently shaking the vial to obtain a homogeneous suspension.
2. Add 300 µl of the slurry (3 mg of beads) to a 1.5-ml tube.
3. Place the tube in the MPC and separate for 1 min. Remove the supernatant by aspiration (leaving the tube in the MPC during aspiration).
4. Resuspend the beads in 300 µl of Solution A (Recipe 12), incubate for 1 min before separation, separate with the MPC, and aspirate the supernatant.
5. Repeat step 4.
6. Wash the beads once with 300 µl of Solution B (Recipe 13), incubate for 1 min before separation, separate with the MPC, and aspirate the supernatant.
7. Resuspend the beads in 300 µl of Binding Buffer (Recipe 15).

Immobilization of a biotinylated oligonucleotide onto GenoPrep streptavidin beads

1. Wash the beads twice with 300 µl of Binding Buffer (Recipe 15), incubate each wash for 1 min before separation, separate with the MPC, and aspirate the supernatant.
2. Resuspend the streptavidin beads in 300 µl of Binding Buffer (Recipe 15) to a final concentration of 10 µg/µl.
3. Add 1200 pmoles of biotinylated gp130-specific oligonucleotide per mg of streptavidin-coated beads (this corresponds to 36 µg of the oligonucleotide for 3 mg of beads).
4. Incubate at room temperature for 15 min with gentle rotation.
5. Separate with the MPC and aspirate the supernatant.
6. Wash three times with 300 µl of Binding Buffer (Recipe 15); incubate each wash for 1 min before separation, separate with the MPC, and aspirate the supernatant.
7. Resuspend the streptavidin beads in 300 µl of Washing Buffer (Recipe 16) to a final concentration of 10 µg/µl.

Preparation of the cell lysates

1. Grow the cells from step 8b in "Colony selection and clone maintenance and storage" until 70 to 95% confluent.
2. Remove the medium and wash the cells with 100 µl of PBS.

   Note: The PBS wash is optional.
3. Add 100 µl of RLT Buffer (RNeasy Kit) with 1% β-mercaptoethanol and transfer to an RNAse-free 1.5-ml tube.

   Note: The RLT buffer is stable for one month after the addition of 1% β-mercaptoethanol.
4. Homogenize the sample using a QIAshredder spin column.
5. Add 400 µl Binding Buffer (Recipe 15) and transfer to a new 1.5-ml tube.

   Note: If the lysates are to be processed later, they can be stored at –20°C or –70°C. Frozen lysates should be thawed by incubating for 10 min at 37°C to completely dissolve salt crystals.

Isolation of prey-specific poly(A)⁺ mRNA

1. Carefully resuspend the biotinylated oligo-linked beads and add 5 µl of the beads to the diluted lysate.
2. Incubate at room temperature for 15 min with gentle rotation.
3. Separate with the MPC and aspirate the supernatant.

4. Wash three times with 300 μl of Washing Buffer (Recipe 16); incubate each wash for 1 min before separation, separate with the MPC, and aspirate the supernatant.

   Note: Take care to resuspend the beads very gently.

5. Elute the bound mRNA by incubating the beads in 30 μl of RNase-Free Distilled Water (Recipe 14) for 2 min at 65°C.
6. Separate with the MPC and transfer the supernatant to a new 1.5-ml tube.
7. Use 5 μl for RT-PCR and store the rest at −80°C.

RT-PCR, purification, and sequencing

1. Set up the reaction mix as described in the protocol of the OneStep RT-PCR kit, including 200 ng of each of the forward gp130-specific primer (5′-GGCATGGAGGCTGCGACTG-3′) and the reverse 3′ LTR-specific primer (5′-TCGTCGAC-CACTGTGCTGGC-3′) and 5 μl of the mRNA template.
2. Program the thermal cycler according to the conditions described in the protocol of the OneStep RT-PCR kit. PCR amplification conditions are as follows. 30 cycles of: 94°C for 1 min; 70°C for 1 min; and 72°C for 2 min.

   Note: Start the RT-PCR program while the sample tubes are still on ice. Wait until the thermal cycler has reached 50°C before placing the tubes in the cycler.

3. Separate the PCR samples on an agarose gel.
4. Isolate the candidate bands and purify the DNA.
5. Determine their sequences by cycle sequencing using a gp130-specific primer (for example, 5′- GAGGCTGCGACTGAT-GAAG-3′).

Troubleshooting

In analytical experiments, the reporter gene signal can be low. In some cases, swapping of bait and prey proteins may lead to higher reporter induction in analytical MAPPIT experiments. This difference may be explained by a sterical configuration leading to better activation of the JAK-STAT pathway.

In both analytical and screening applications of MAPPIT, the bait proteins should not activate STAT3 either directly or indirectly, because detection of the test interaction is STAT3-dependent. Although a low activation of STAT3 may not induce significant luciferase levels in analytical MAPPIT experiments, it may lead to colony formation in screening experiments. Therefore, it is essential before screening to determine the optimal selective puromycin concentration. It is of note that the rPAP1 promoter is also responsive to activated STAT5. This does not interfere with MAPPIT experiments in HEK293-type cells, because the endogeneous STAT5 levels are very low.

Because multiple prey cDNA integrants can be obtained in a single colony, subcloning of prey into the pMG1 plasmid vector is required for individual testing and confirmation of the interaction.

Nonspecific binding to the LR-F3 domain has so far only been observed for the suppressor of cytokine signaling 2 (SOCS-2) prey, when overexpressed using the pMET7 vector. This was not seen when expression was under control of the SV40 early promotor as in the pSV-SPORT vector. Thus, nonspecific binding may be decreased by reducing the expression level of the prey.

Notes and Remarks

MAPPIT functions in mammalian cells, and, therefore, opens the possibility of studying protein-protein interaction in intact cells, under near physiological conditions. Thus, the effects of various regimens (for example, extracellular signals, drug treatment, or cell stress) on a given interaction can be monitored, or detailed structure-function analyses can be performed. Furthermore, some bait polypeptides (ideally, but not necessarily, limited to signaling molecules associated with the JAK-STAT pathway), may act as substrates for JAK activity and may thus become phosphorylated after receptor activation. Using heteromeric receptor complexes, we were also able to demonstrate serine phosphorylation-dependent interactions in the transforming growth factor-β (TGF-β) signaling pathway, whereby a serine-threonine kinase was linked to one of the receptor chains, and the substrate was cloned to the other chain (*11*).

The precise relationship between analytical use and screening performance of MAPPIT has not yet been rigorously established. We have observed detectable binding of various signaling molecules, including cytokine-inducible SH2-containing protein (CIS), SOCS-2, SH2-containing phosphotyrosine phosphatase 2 (SHP-2), phospholipase Cγ (PLCγ), and the p85 subunit of phosphatidyli-

nositol 3-kinase (PI3K) with their tyrosine-phosphorylated recruitment sites, the serine-phosphorylation-dependent Smad3-Smad4 and Smad3-Smad interacting protein 1 (SIP1) interactions, as well as modification-independent interactions such as p53-SV40 Large T (SVT) or activin-like kinase 4 (ALK4)-FK506-binding protein 12 (FKBP12) (*6*, *10*, *11*). Screening experiments clearly allowed cloning of SOCS family members, but additional experiments are required to determine the precise threshold limits of this colony-based selection approach.

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Design and Use of a Mammalian Protein-Protein Interaction Trap (MAPPIT)

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