Methods 53 (2011) 453–459

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Extracting gene function from protein–protein interactions using Quantitative BAC InteraCtomics (QUBIC)

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ARTICLE INFO

Article history:
Accepted 17 December 2010
Available online 22 December 2010

Keywords:
QUBIC
Protein–protein interaction
Quantitative mass spectrometry
Proteomics
BAC transgeneomics
Label-free quantification
SILAC

ABSTRACT

Large-scale proteomic screens are increasingly employed for placing genes into specific pathways. Therefore generic methods providing a physiological context for protein–protein interaction studies are of great interest. In recent years many protein–protein interactions have been determined by affinity purification followed by mass spectrometry (AP–MS). Among many different AP–MS approaches, the recently developed Quantitative BAC InteraCtomics (QUBIC) approach is particularly attractive as it uses tagged, full-length baits that are expressed under endogenous control. For QUBIC large cell line collections expressing tagged proteins from BAC transgenes or gene trap loci have been developed and are freely available. Here we describe detailed workflows on how to obtain specific protein binding partners with high confidence under physiological conditions. The methods are based on fast, streamlined and generic purification procedures followed by single run liquid chromatography–mass spectrometric analysis. Quantification is achieved either by the stable isotope labeling of amino acids in cell culture (SILAC) method or by a 'label-free' procedure. In either case data analysis is performed by using the freely available MaxQuant environment. The QUBIC approach enables biologists with access to high resolution mass spectrometry to perform small and large-scale protein interactome mappings.

1. Introduction

Almost all cellular processes rely on protein–protein interactions that can be easily disturbed by biological stimuli or during disease. For example, tumorigenesis is often the result of gene mutations leading to aberrant signaling cascades due to altered protein–protein interactions in signaling cascades. Rapid and unbiased identification of these altered protein–protein interactions is thus essential for unraveling the underlying molecular mechanisms. For this, affinity purification of protein complexes followed by mass spectrometry (AP–MS) is presently the method of choice. This method has already been successfully applied for the characterization of the yeast interactome [1–4].

Standard AP–MS methods suffer from two major problems. First, the mass spectrometric measurements are usually performed in a non quantitative manner. This makes it difficult to distinguish true interaction partners from background proteins that bind to the affinity matrix and as a result the approach is often associated with high false positive rates. Addressing the high false positive rates has previously required tandem affinity purification – usually combined with gel electrophoresis – to obtain visually distinct protein bands [5]. Since this procedure requires large amounts of starting material it cannot be easily scaled up to mammalian systems. Moreover, since the purification protocol is intricate and involves numerous steps, transient interaction partners are usually lost. The second problem is that most current protein interaction studies in mammalian cells still rely on tagged bait proteins overexpressed from exogenous promoters, a strategy that frequently generates protein localization and interaction artifacts. Furthermore, the modification state of overexpressed proteins may be different from the endogenous protein and this may also affect protein interactions.

The recently described Quantitative BAC InteraCtomics (QUBIC) strategy circumvents these problems as it is based on (i) the expression of tagged full length bait proteins under physiological conditions, (ii) single step immunopurification (IP) and (iii) quantitative mass spectrometry [6]. Tagged bait proteins can be created in all transfectable cell lines by BAC transgeneomics [7–9] or alternatively, by gene trapping [10,11] or targeting in embryonic stem cells [12]. Detailed protocols for the different protein tagging methods are given in this volume by Schnütgen et al and Hofemeister et al (pages 347 and 437, respectively).

All the tagging methods employ the enhanced green fluorescence protein (eGFP) or its derivatives because excellent generic antibodies are available for purification and because the eGFP-tagged proteins are amenable for life cell imaging [9,13]. In all QUBIC approaches described here, tags are introduced directly into the genomic loci, which normally express the bait protein of interest. This ensures cell-type specific processing and regulation of the bait protein.

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1046-2023/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymeth.2010.12.016

To optimize the identification of transient interaction partners, immunopurification procedures require speed and sensitivity which is achieved by avoiding harsh conditions, such as buffers containing high salt or detergent. Here, we describe a single-step protocol for eGFP-tagged protein purification using magnetic beads combined with flow-through, column-based purification and in-column tryptic digestion [6]. QUBIC can be easily adapted to other protein tags.

Quantitative proteomics can efficiently distinguish true interaction partners from background binders [14–17]. With QUBIC this is achieved either by stable isotope labeling of amino acids in cell culture (SILAC) [18], which is highly accurate [19], or by a label-free procedure and analysis is performed with the MaxQuant software suit [20,21]. In both cases pull-downs from the eGFP-tagged cell line are compared to pull-downs from the corresponding untagged wildtype cell line. While peptides from background binders give the same relative signal intensities in both purifications, bait interacting proteins are much more abundant in the pull-downs from the transgenic line expressing the tagged bait protein.

In summary, QUBIC is a novel and generic tag based proteomics method that is reliable and highly specific without requiring extensive protein purification steps. Its sensitivity enables bait recovery under physiological conditions. For this reason it can be applied for characterizing dynamic interactomes that transiently assemble in cells undergoing various phenotypic changes [6,22]. Below, we outline an optimized protocol for interactome analysis using eGFP-tagged bait proteins. The technique is simple and cost effective, hence useful for both small and large scale protein interaction screens.

2. Materials and supply list

2.1. Cell culture

Label-free cells are cultured in standard cell culture media. SILAC media contains dialyzed serum with a cutoff of 10 kDa and heavy arginine and lysine.

As an example we describe SILAC media as standard DMEM medium for HeLa cell culture:

Component	Company	Ordering-#
DMEM (4.5 g/L glucose, -lysine, -arginine)	Invitrogen	10829018
10% fetal bovine serum, dialyzed with a cutoff of 10 kDa	Invitrogen	26400044
100 U/ml penicillin/streptomycin	Invitrogen	15140122
73 μg/ml Lysine light ($C^{12}N^{14}$)	Sigma	L8662
Or 73 μg/ml lysine heavy ($C^{13}N^{15}$)	CIL	CNLM-291-H-1
42 μg/ml Arginine light ($C^{12}N^{14}$)	Sigma	A6969
Or 42 μg/ml arginine heavy ($C^{13}N^{15}$)	CIL	CNLM-539-1

2.2. Immunopurification

2.2.1. Equipment

Component	Company	Ordering-#
Handmagnet or MultiMACS	Miltenyi Biotec	130-042-602, 130-091-937
μColumns	Miltenyi Biotec	130-042-701

2.2.2. Special reagents

Component	Company	Ordering-#
μMACS anti-GFP	Miltenyi Biotec	10829018
IGPAL-CA-630	Sigma	I8896
Protease inhibitors, EDTA-free	Roche	11836153001
Benzonase	Merck	70746-3
Trypsin	Promega	V511C
Sodium butyrate	Fluka	19364
Sodium fluoride	Sigma	201154
Glycerol 2-phosphate	Sigma	G6376
Sodium orthovanadate	Sigma	S6508

2.3. LC–MS/MS

The analysis software MaxQuant currently only works for raw data acquired on an LTQ-FT, LTQ-Orbitrap or LTQ-Orbitrap Velos (all Thermo Fisher Scientific). This system needs to be coupled online to a nano-LC system (e.g. EASY-nLC II from Proxeon Biosystems, now Thermo Fisher Scientific). Columns can be pulled and packed in-house (e.g. fused-silica emitter with an inner diameter of 75 mm (Proxeon) packed with RP ReproSil-Pur C_{18}-AQ 3 mm resin (Dr. Maisch) or ordered for example from Proxeon.

2.4. Data analysis

The raw data is processed by MaxQuant. This software is available for free download at http://www.maxquant.org. The download includes the Andromeda search engine implemented in MaxQuant. The downstream Perseus analysis software required for determining the significance of specific protein–protein interactions is available for download at http://www.perseus-framework.org.

3. Methods

3.1. Cell culture for SILAC and label-free QUBIC

QUBIC is a quantitative proteomics method. Protein quantification is achieved by comparing relative intensities of the same peptide in the mass spectrometer (Fig. 1). This can either be done by stable isotope labeling of amino acids in cell culture (SILAC) [18], in which case both peptides appear in the same mass spectra, or by label-free protein quantification, in which case the peptides appear in different LC–MS/MS runs. Although not described here, QUBIC can in principle also be performed with chemical labeling techniques (for reviews see [23,24]). Label-free protein quantification enables the comparative analysis of proteomes expressed by cells under manifold conditions without requiring any special pretreatment of the cells. It is therefore a preferred method for protein–protein interaction mapping, especially when multiple baits are used. Conversely, SILAC is approximately five times more accurate than the label-free approach and therefore more appropriate for detecting minor changes (<4-fold) such as those occurring in dynamic protein interaction mappings. With SILAC, up to three distinct conditions can be compared in a single experiment using two different amino acid labels in addition to the unlabeled amino acids.

QUBIC relies on the comparative quantification of proteins pulled down by an anti-eGFP antibody from transgenic (bait expressing) and non-transgenic control cell lines. As illustrated in