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iCLIP: Protein–RNA interactions at nucleotide resolution

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ABSTRACT

RNA-binding proteins (RBPs) are key players in the post-transcriptional regulation of gene expression. Precise knowledge about their binding sites is therefore critical to unravel their molecular function and to understand their role in development and disease. Individual-nucleotide resolution UV crosslinking and immunoprecipitation (iCLIP) identifies protein–RNA crosslink sites on a genome-wide scale. The high resolution and specificity of this method are achieved by an intramolecular cDNA circularization step that enables analysis of cDNAs that truncated at the protein–RNA crosslink sites. Here, we describe the improved iCLIP protocol and discuss critical optimization and control experiments that are required when applying the method to new RBPs.

1. Introduction

Post-transcriptional regulation critically contributes to the ability of cells to adjust gene expression in the face of a changing external or internal environment [1]. RNA-binding proteins (RBPs) are the primary regulatory factors of the various post-translational stages, including alternative splicing, polyadenylation, mRNA localization, translation and degradation [2]. Their RNA binding is mediated by modular RNA-binding domains (RBDs), such as the RNA recognition motif (RRM), hnRNP K-homology domain or zinc fingers (Znf) [3]. Although many RBDs recognize RNA in a sequence-specific manner, sequence information is not sufficient to reliably predict RBP binding sites throughout the transcriptome. In particular, RBPs cooperate and compete when binding to RNA; therefore it is crucial to study protein–RNA interactions in the cellular environment.

The first approaches to investigate protein–RNA complexes *in vivo* employed affinity purification or immunoprecipitation combined with microarray analysis (RIP-CHIP). However, these approaches were prone to identifying non-physiologic or indirect interactions and their low resolution made it difficult to narrow down actual binding sites [4,5]. Development of *in vivo* UV-crosslinking and immunoprecipitation (CLIP) enabled the study of protein–RNA interactions with high positional resolution and specificity. Combined with high-throughput sequencing, CLIP became the standard tool for the genome-wide analysis of protein–RNA interactions [6].

In the original CLIP approach, reverse transcription needs to proceed from a universal 3′ ligated adapter to a universal 5′ ligated adapter, since both adapters are required for PCR amplification. However, in over 80% of cases, the reverse transcriptase stalls at the short polypeptide left at the UV-induced crosslink site, resulting in truncated cDNAs that lack the 5′ adapter, and are therefore not amplified in CLIP [7]. We previously developed individual-nucleotide resolution CLIP (iCLIP), which enables PCR amplification of truncated cDNAs, and thereby identifies protein–RNA crosslink sites with nucleotide resolution (Fig. 1) [8]. iCLIP has been successfully used to study the function of RBPs in alternative splicing [8–13], alternative polyadenylation [14], RNA methylation [15] and mRNA stability [16]. It determined high-resolution RNA splicing maps of different RBPs, which enabled to assess how the position of RBP binding around alternative exons determines their splicing function [8–13,17]. Importantly, in addition to identifying the RBP binding sites of an RBP, iCLIP can also quantitate

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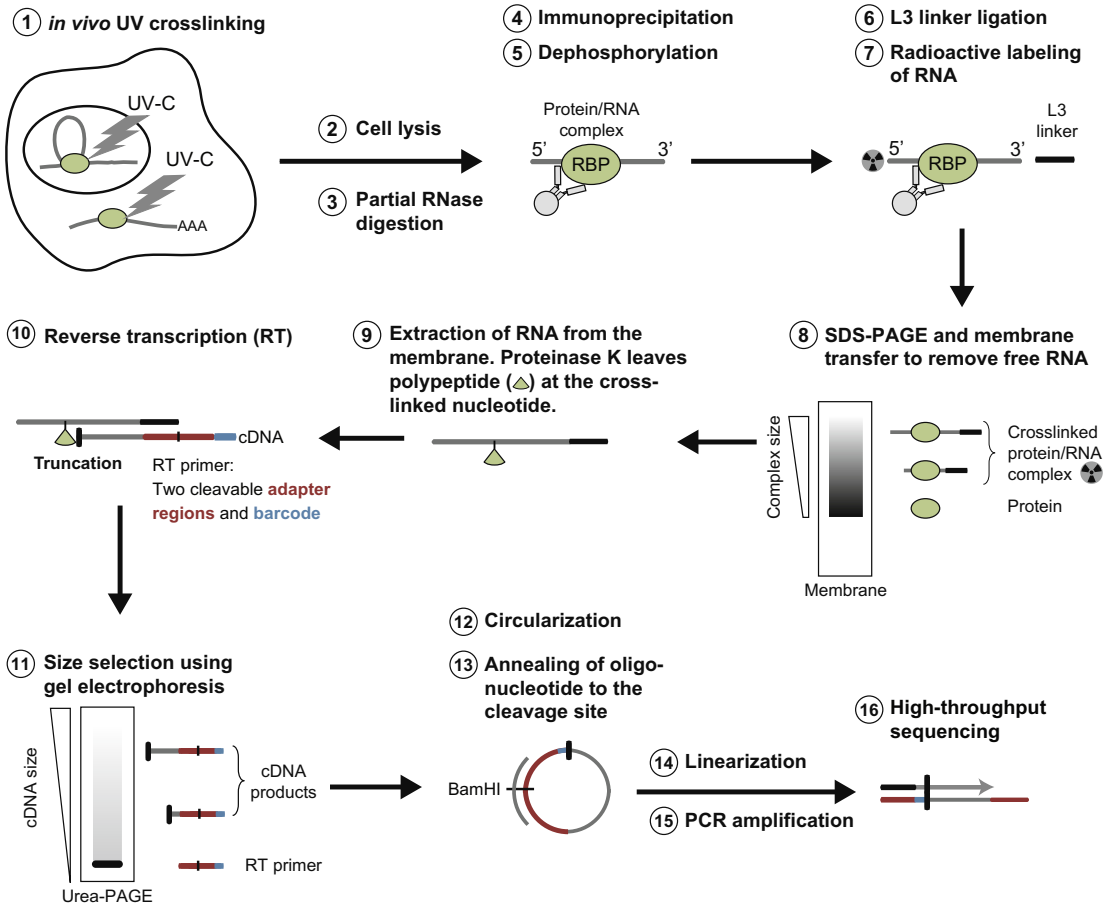

Fig. 1. Schematic representation of the iCLIP procedure identifying RNA–protein interactions in intact cells. Cells are irradiated with UV-C light on ice, leading to formation of a covalent bond between protein and RNA. This is followed by partial RNase digestion and an immunoprecipitation with protein-specific antibodies. For the library preparation and visualization, the RNA is dephosphorylated, a 3′ end adapter is ligated and the 5′ end is radioactively labeled. The complexes are separated by SDS–PAGE and isolated from a nitrocellulose membrane according to the expected size. The protein is then digested by proteinase K, and reverse transcription (RT) is performed truncating at the remaining polypeptide. The RT primer introduces two cleavable adapter regions and barcode sequences. The free RT primers are removed by size selection and circularization of the cDNA is carried out. Linearization generates suitable templates for PCR amplification. In the last step, high-throughput sequencing generates reads in which the barcode sequences are immediately followed by the last nucleotide of the cDNA.

genome-wide changes in protein–RNA interactions; for instance, it was demonstrated that the splicing factor U2AF65 gains access to hundreds of Alu elements after knockdown of hnRNP C, which prevents their erroneous recognition under normal conditions [18].

The iCLIP protocol starts with UV irradiation, which forms covalent bonds at sites of protein–RNA interactions and thereby preserves the *in vivo* binding pattern (Fig. 1, step 1). Next, the cells are lysed and the RNAs are partially digested to obtain RNA fragments in an optimal size range (Fig. 1, steps 2 and 3). After immunoprecipitation of the protein–RNA complexes, the RNA is dephosphorylated, an adapter is ligated to the 3′ end of the RNA and the 5′ end is radioactively labeled (Fig. 1, steps 4–7). SDS–PAGE and transfer to nitrocellulose membrane enable to remove free RNA and to stringently purify the crosslinked protein–RNA complexes (Fig. 1, step 8). The RNA is recovered from the nitrocellulose membrane by digesting the protein with proteinase K, which leaves a polypeptide at the crosslink site (Fig. 1, step 9). The RNA is then reverse transcribed into cDNA, which most often truncates at the polypeptide remaining at the crosslink site (Fig. 1, step 10). Size selection of the cDNA removes free reverse transcription (RT) primers, and is followed by cDNA circularization, which attaches the second adapter to the 3′ end of cDNA (Fig. 1, steps 11–13). Restriction enzyme digestion linearizes the cDNA before PCR amplification (Fig. 1, steps 14 and 15). To increase the quantitative power of the method, the reverse transcription primers contain a randomized sequence (random barcode), which enables computational filtering of artifacts caused by variable PCR amplification of the cDNAs [19]. After high-throughput sequencing, the barcode sequence precedes the cDNA sequence. Truncated cDNAs represent over 80% of the cDNA library, and after mapping their sequence to the genome, the position of the preceding nucleotide corresponds to the crosslinking site [7].

In the following section, we provide a detailed description of required materials and experimental procedures of the iCLIP protocol. In particular, we introduce important controls and optimization steps including some example experiments. Throughout the protocol, we point out critical steps and give advice on how to optimize them.