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Protocol

DyNAbind® 10 Million Compound DNA-Encoded Library DEL Kit Use Guide

Product Number - DYNA002

On behalf of our entire team at DyNAbind, we thank you for purchasing this DNA-Encoded Library kit! Inside, 10 million unique DNA-tagged molecules are ready to be deployed against your target protein. This kit has been designed to be usable by nearly anyone with common biochemistry lab experience. However, you will need the following skills and equipment:

- Benchtop centrifuge
- Standard PCR
- Realtime PCR
- Gel Electrophoresis and Purification
- Nanodrop or other spectrophotometer for optical DNA quantification

In addition, certain PCR primers are required for working up the results of the selections. **Please carefully read this entire document before beginning to ensure that the necessary reagents, primers and equipment are available.**

Here's an overview of the process. First, protein will be immobilized on solid support beads and selection will be carried out with the library. After completion of selection, the eluted DEL samples must be prepared for sequencing to reveal the identities of the top binding compounds. First, real-time PCR is used to determine the appropriate number of PCR cycles for workup. Next, traditional PCR is used to amplify the selected DNA codes and add some necessary DNA for Illumina sequencing, including the Molecular Identifier (MID) DNA tags which will identify each experiment (e.g. condition X, condition Y, control etc.) in the sequencing data later. Then after gel electrophoresis, DNA is purified, and a final PCR step is performed to prepare samples for sequencing. After a last gel electrophoresis and extraction, samples can be submitted for sequencing. Let's get started! We have provided two options in the protocol to allow for working with either a His-tagged (1a) or a biotinylated (1b) protein. Other methods of attaching a protein to beads can be used but may require additional optimization.

Regardless of which solid support immobilization strategy you use, **we strongly recommend the use of a control experiment against a blank solid support**. In any DEL selection experiment, some amount of binding to the solid support should be expected. Submitting a control experiment sample will allow these compounds to be filtered out from the experimental samples, increasing your chances of success.

1a) Selection With the DEL Using a His-Tag Protein

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In this first stage, we'll immobilize a His-tagged protein on His-tag pulldown solid support beads and screen the library to select binding structures. It's important to remember that for each target and condition set used, a library aliquot should also be run against non-functionalized beads as a negative control.

His-Tag Pulldown Bead Protocol

Prepare the following stocks of working buffers:

Important: these are neutral starting points for buffer composition. Adjust them to suitable pH and salt values for your protein. Also consider where possible ideal temperatures for stability of your protein during the selection process. Critical buffer components are indicated with bold text.

Note: When working with solid support beads, consult the manufacturer's specifications and adapt the protocol accordingly for, e.g. loading beads and pulling down with a magnet rack.

- PBS: 50 mM sodium phosphate, 100 mM NaCl, pH 7.4
- Washing buffer (PBS-T): PBS supplemented with **0.05% TWEEN-20**
- DEL buffer (PBS-T-HS): PBS supplemented with **0.05% TWEEN-20** and **0.01 mg/ml herring sperm DNA**
- Elution buffer: **360 mM imidazole**, dissolved in 50 mM Tris-HCl pH 8.0, 150 mM NaCl and 0.05% Tween-20

1. Place 10 to 25 µL of His-tag pulldown beads (for example holding up to 40 µg of 20 to 50 kDa protein) in a 1.5 mL protein low-binding microcentrifuge tube.
2. Wash with 1 mL of Washing Buffer, pull the beads down in a magnetic rack and carefully remove the supernatant without touching the beads. Repeat two more times.
3. Prepare 100 µL of His-tagged protein solution in PBS or other protein-suitable buffer. Protein amount should be at 1.5 times excess the bead loading capacity.
4. Resuspend the beads in the protein solution in a protein low-binding microcentrifuge tube and incubate for 30 minutes with gentle rocking in a mixer.
5. Towards the end of the incubation for Step 4, prepare the DEL Solution by dissolving the DEL pellet in 100 µL of DEL Buffer, vortexing until the pellet is dissolved, typically about 20 s, and then briefly spinning down in a benchtop centrifuge.
6. Carefully remove the supernatant from the beads of Step 4 without touching them. Add 200 µl of Washing Buffer to the beads, carefully transfer the beads to a 1.5 mL DNA low binding microcentrifuge tube, pull the beads down in a magnetic rack, and carefully remove the supernatant.
7. Resuspend the beads in 100 µL of DEL Solution from Step 5 and incubate the tube for 2 hours with gentle rocking in a mixer.
8. Remove the DEL Solution and wash three times with 200 µL of Washing Buffer, pulling the beads down in a magnetic rack, and carefully removing supernatant each time. Change the pipette tip and transfer to a new DNA low binding tube for each washing step.
9. Resuspend in 100 µl of Elution Buffer and then transfer to a new DNA low binding microcentrifuge tube.
10. Incubate for 10 minutes with gentle rocking in a mixer.
11. Pull down the beads in a magnetic rack and transfer the supernatant containing the DNA-tags to a new DNA low binding tube.
12. Continue to section 2.

1b) Selection With the DEL Using a Biotinylated Protein

In this first stage, we'll immobilize a biotinylated protein on streptavidin solid support beads and screen the library to select binding structures. It's important to remember that for each target and condition set used, a library aliquot should also be run against non-functionalized beads as a negative control.

Streptavidin Sepharose Bead Protocol

Prepare the following stocks of working buffers:

Important: these are neutral starting points for buffer composition. Adjust them to suitable pH and salt values for your protein. Also consider where possible ideal temperatures for stability of your protein during the selection process. Critical buffer components are indicated with bold text.

Note: When working with solid support beads, consult the manufacturer's specifications and adapt the protocol accordingly for, e.g. loading beads and spinning down in a centrifuge.

- PBS: 50 mM sodium phosphate, 100 mM NaCl, pH 7.4
- Washing buffer (PBS-T): PBS supplemented with **0.05% TWEEN-20**
- Blocking buffer: PBS supplemented with **0.05% TWEEN-20** and **100 µM D-biotin**
- DEL buffer (PBS-T-HS): PBS supplemented with **0.05% TWEEN-20** and **0.01 mg/ml herring sperm DNA**
- Elution buffer: **10 mM Tris**, **pH 8.5**, supplemented with **0.05% TWEEN-20**

1. Place 10 to 30 µL of streptavidin sepharose beads (for example holding up to 40 µg of 20 to 50 kDa protein) in a 1.5 mL protein low-binding microcentrifuge tube.
2. Wash with 1 mL of Washing Buffer, centrifuge the beads down and carefully remove the supernatant without touching the beads. Repeat two more times.
3. Prepare 100 µL of biotinylated protein solution in PBS or other protein-suitable buffer. Protein amount should be at 1.5 times excess the bead loading capacity.
4. Resuspend the beads in the protein solution and incubate 30 minutes with gentle rocking in a mixer, and then carefully remove the supernatant without touching the beads.
5. Add 200 µl of Blocking Buffer, incubate for 5 minutes with gentle rocking in a mixer, and then carefully remove the supernatant without touching the beads. Repeat once more.
6. During the second incubation for Step 5, prepare the DEL Solution by dissolving the DEL pellet in 100 µL of DEL Buffer, vortexing until the pellet is dissolved, typically about 20 s, and then briefly spinning down in a benchtop centrifuge.
7. Add 200 µl of Washing Buffer to the beads from Step 5, carefully transfer the beads to a 1.5 mL DNA low binding microcentrifuge tube, spin down in a centrifuge, and carefully remove the supernatant.
8. Resuspend the beads in 100 µL of DEL Solution from Step 6 and incubate for 2 hours with gentle rocking in a mixer.
9. Remove the DEL Solution and wash three times with 200 µL of Washing Buffer, spinning down in a benchtop centrifuge and carefully removing supernatant each time. Change the pipette tip and transfer to a new DNA low binding tube for each washing step.
10. Resuspend in 100 µl of Elution Buffer and then transfer to a new DNA low binding microcentrifuge tube.
11. Heat at 95°C for 10 minutes with gentle rocking.
12. Briefly spin the beads down in a benchtop centrifuge and transfer the supernatant containing the DNA-tags to a new DNA low binding tube.

13. Continue to section 2.

2) Realtime PCR

As the amount of bound structures and recovered DNA can vary depending on the target and selection conditions, it is important to first quantify the DNA present so the following workup steps can be performed correctly. For a list with all the primers used in the protocol, see Appendix B.

The following primers are required:

Primer	**Sequence**
A1	TCACTTCTGGACACAATCGCTTGC
A2	GGAGGTCTTGCAGACAGAGGA

Set up the first PCR mixture according to the following table:

Component	**10 µl Reaction**
qPCR Master mix 2x	5 µL
Primer A1 5 µM	1 µL
Primer A2 5 µM	1 µL
Nuclease-free water	2 µL
Eluted selection DNA 1:10 diluted	1 µL

Run the real-time PCR according to the following program:

STEP	**TEMP**	**TIME**

1x	95°C	60 seconds
35 Cycles	98°C 62°C 72°C	15 seconds 30 seconds 30 seconds
(Optional meltcurve)	60-95°C	
10 min	72°C	

Once the real-time PCR is complete, the readout is used to determine the optimal cycle number for preparative PCR. The ideal cycle number is the highest one still clearly in the exponential PCR phase, as indicated in Figure 1 below.

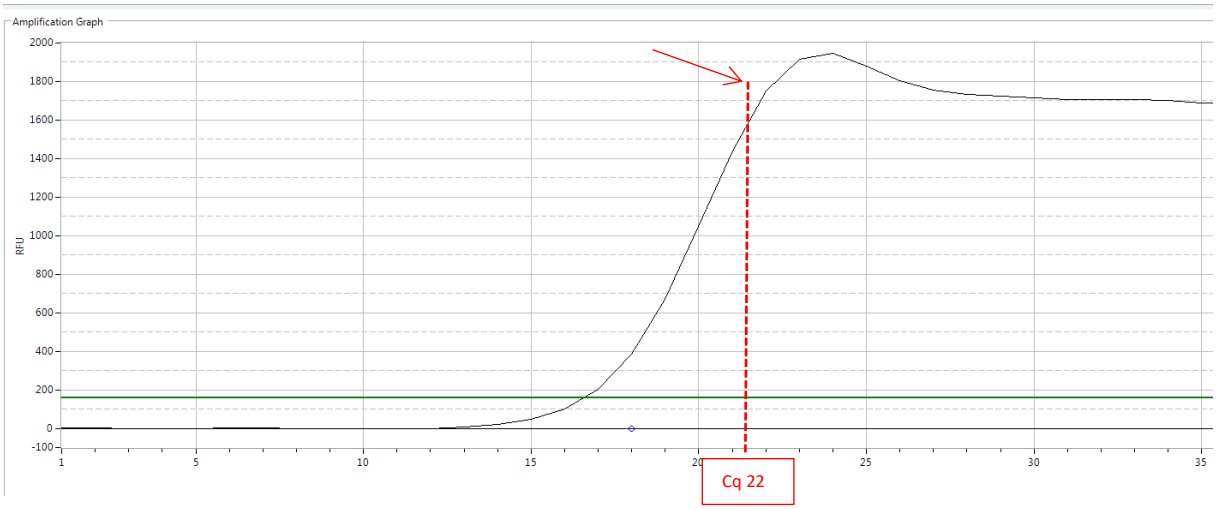

Figure 1. Determining the cycle number from qPCR for preparative PCR amplification.

3) Preparative PCR 1

Next, we'll perform preparative PCR on each eluted product to amplify the contents and introduce the MID tags which will be used to mark and separate the different experiments during analysis of the sequencing.

As in the real-time PCR step, one PCR run will be performed for each selection condition. Each condition requires a unique MID. Thus, different versions of Primer B2 below will need to be produced to fit your needs. After collecting the Illumina sequencing data, the MIDs will be entered in the analysis portal on the Sigma-Aldrich site for decoding the sequences associated with a particular sample. The analysis requires that all the MIDs are the same length. Appendix A lists 15 MID sequences that are predesigned which may be usable, but check with your sequencing provider to confirm their usability or for advice on designing MIDs. Appendix B lists all the primers for the protocol including an example of five B2 primers containing possible MIDs. Appendix B also goes into more detail about the functional elements of the primers.

Note: When only two MIDs are used in a single sequencing flow cell, measures should be taken to ensure appropriate sample diversity for good quality Illumina sequencing. Consult your sequencing provider to determine the optimal methodology. Potential solutions include spiking in PhiX Control sequences or introducing a degenerated cassette in Primer B2 immediately before the MID sequences. If the degenerated cassette option is preferred, Appendix D indicates how primers may be modified.

The following primers are required:

Primer	**Sequence**
B1	CAAGCAGAAGACGGCATACGAGATTCACTTCTGGACACAATCGCTTGC
B2	ACACTCTTTCCCTACACGACGCTCTTCCGATCT**MID**GGAGGTCTTGCAGACAGAGGA

For each condition tested, set up the PCR mixture according to the following table:

Component	**50 µl Reaction**	**Final Concentration**
Nuclease-free water	to 50 µl	
5X HF PCR Buffer	10 µL	1X
10 mM dNTPs	1.25 µL	250 µM
Primer B1 5 µM	6 µL	0.6 µM
Primer B2 (w/ MID) 5 µM	6 µL	0.6 µM
Eluted Selection DNA 1:10 diluted	1 µL	
High Fidelity Polymerase	1 U	1.0 units

Run the PCR according to the following program, using the cycle number determined previously by the real-time PCR experiment:

STEP	TEMP	TIME
Initial Denaturation	98°C	60 seconds
15-25 Cycles	98°C 62°C 72°C	30 seconds 60 seconds 30 seconds
Final Extension	72°C	10 minutes
Hold	4°C	

4) Agarose Gel Electrophoresis

Next, the PCR product is purified via agarose gel electrophoresis.

A 2.0% agarose gel should be used with either TAE or TBE buffer. Use a gel that can accommodate all 50 µL of the PCR amplification product. Use a DNA ladder covering a 100 to 500 bp range to track the product bands.

The products are expected to be 146 bp.

5) Agarose Gel Purification

The next step is to extract the purified PCR products from the agarose gel. This step can be performed with any agarose purification kit you prefer.

Tip: After dissolving the agarose gel in your kit's dissolving buffer, mix the dissolved sample with isopropanol 1:1 for better DNA yield.

Afterward, quantify each sample via Nanodrop or equivalent spectrophotometer. You may need to dilute the sample if it is outside the linear range.

6) Preparative PCR 2

A final PCR protocol is now performed to prepare the samples for Illumina Sequencing. Consult with your sequencing provider to determine the appropriate loading capacity and number of samples which can be pooled together for submission. It is recommended to collect a sequence length of 90 bp and a depth of at least 3 million reads per MID with the single read option. Based on these considerations, the quantified 1st PCR products can be run separately or pooled together in one or more groups. The final concentration of a single DNA sample should be 15 nM. If

samples are combined, they should be pooled together at equimolar concentration to a final concentration of 15 nM. At this stage, each sample will use the same pair of primers.

The following primers are necessary:

Primer	**Sequence**
C1	CAAGCAGAAGACGGCATACGAGAT
C2	AATGATACGGCGACCACCGAGATCTACACACACTCTTTCCCTACACGAC

Prepare the PCR mixture according to the following table:

Component	**50 µl Reaction**	**Final Concentration**
Nuclease-free water	to 50 µl	
5X HF PCR Buffer	10 µl	1X
10 mM dNTPs	1.25 µl	250 µM
Primer C1 5 µM	6 µl	0.6 µM
Primer C2 5 µM	6 µl	0.6 µM
Template DNA 15 nM	1 µL	0.3 nM
HF Polymerase	0.5 µl	1.0 units

Run each PCR according to the following program:

STEP	**TEMP**	**TIME**
Initial Denaturation	98°C	60 seconds
20 Cycles	98°C 62°C 72°C	30 seconds 60 seconds 30 seconds
Final Extension	72°C	10 minutes
Hold	4°C	

7) Agarose Gel Electrophoresis

Next each amplified sequencing-ready construct must be separated with another agarose gel electrophoresis run.

A 2.0% agarose gel should be used with either TAE or TBE buffer. Use a gel that can accommodate all 50 µL of the PCR amplification product. Use a DNA ladder covering a 100 to 500 bp range to track the product bands.

The product of each reaction is expected to be 175 bp.

8) Agarose Gel Purification

Finally, each DNA construct must be extracted from the agarose gel. As in step 5, follow the directions of your preferred agarose gel purification kit.

Tip: After dissolving the agarose gel in your kit's dissolving buffer, mix the dissolved sample with isopropanol 1:1 for better DNA yield.

Afterward, quantify each sample via Nanodrop or equivalent spectrophotometer. You may need to dilute the sample if it is outside the linear range.

Dilute each sample for Illumina sequencing based on the recommendation of your sequencing provider. Submit each sample for unpaired sequencing – paired sequencing is not supported. Once the sequencing data is obtained, the FASTQ files can be submitted to the DEL NGS Analysis Portal (https://www.sigmaaldrich.com/DELanalysis). Please see the Usage Instructions for the portal. They can be found on the product detail page for the kit on the Sigma-Aldrich website.

Appendix A: MIDs

These 15, 12-base pair MIDs have been designed with an equal representation of the 4 bases at each position. These can be used for the MIDs in primer B2 in section 3. **When choosing the different MIDs, maintain the highest sequence diversity, especially in the first 4 bases.**

TCGAACCTAGAC
ATTGAGAGCGAG
CGATCATATCGA
GCTCGCGCTGAT
TAGCACGAACTC
AGAGGCGATGCG
CGCGTATATCGC
GATCAGAGCATA
TCGCTGAGACTC
ATACGCCCTTCT
CACACATCGAGC
GAGTTTCTCGAT
TATGATCTACTG
AGTCCAAAGTAG
GCGTCATAGTAT

Appendix B: Primers

This appendix lists all the primers for the protocol including an example of five B2 primers containing possible MIDs. Each sample should have a separate MID. Primers A1 and A2 are used to quantify the DNA. Primer B1 is used to introduce one of the sequences for the flow cell binding (italics). Primer B2 is used to introduce the entire multiplexing Read 1 Illumina sequence (underlined) and the MIDs (bold). Primer C2 introduces the other Illumina sequence for flow cell binding (italicized) and contains part of the multiplexing Read 1 Illumina sequence (underlined) that was introduced with primer B2. Primer C1 contains the sequence for flow cell binding (italics) that was already introduced with primer B1. When ordering primers, using unformatted sequences may be preferable for some vendors. Appendix C contains the unformatted primers.

Primer	Sequence
A1	TCACTTCTGGACACAATCGCTTGC
A2	GGAGGTCTTGCAGACAGAGGA
B1	*CAAGCAGAAGACGGCATACGAGAT*TCACTTCTGGACACAATCGCTTGC
B2 with MID1	ACACTCTTTCCCTACACGACGCTCTTCCGATCT**TCGAACCTAGAC**GGAGGTCTTGCAGACAGAGGA
B2 with MID2	ACACTCTTTCCCTACACGACGCTCTTCCGATCT**ATTGAGAGCGAG**GGAGGTCTTGCAGACAGAGGA
B2 with MID3	ACACTCTTTCCCTACACGACGCTCTTCCGATCT**CGATCATATCGA**GGAGGTCTTGCAGACAGAGGA
B2 with MID4	ACACTCTTTCCCTACACGACGCTCTTCCGATCT**GCTCGCGCTGAT**GGAGGTCTTGCAGACAGAGGA
B2 with MID5	ACACTCTTTCCCTACACGACGCTCTTCCGATCT**TAGCACGAACTC**GGAGGTCTTGCAGACAGAGGA
C1	*CAAGCAGAAGACGGCATACGAGAT*
C2	*AATGATACGGCGACCACCGAGATCTACAC*ACACTCTTTCCCTACACGAC

Appendix C: Primers Without Formatting

Here is a list of all of the primers required for the protocol. Formatting has been removed, which may be preferable when ordering.

Primer	Sequence
A1	TCACTTCTGGACACAATCGCTTGC
A2	GGAGGTCTTGCAGACAGAGGA
B1	CAAGCAGAAGACGGCATACGAGATTCACTTCTGGACACAATCGCTTGC
B2 with MID1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCGAACCTAGACGGAGGTCTTGCAGACAGAGGA
B2 with MID2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATTGAGAGCGAGGGAGGTCTTGCAGACAGAGGA
B2 with MID3	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGATCATATCGAGGAGGTCTTGCAGACAGAGGA
B2 with MID4	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCTCGCGCTGATGGAGGTCTTGCAGACAGAGGA
B2 with MID5	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTAGCACGAACTCGGAGGTCTTGCAGACAGAGGA
C1	CAAGCAGAAGACGGCATACGAGAT
C2	AATGATACGGCGACCACCGAGATCTACACACACTCTTTCCCTACACGAC

Appendix D: Degenerate primers for Preparative PCR 1

Here are the primers for Preparative PCR 1 which may be used when only two MIDs are included in a single sequencing flow cell and no PhiX Control is added. N indicates a random base.

Primer	**Sequence**
B1	CAAGCAGAAGACGGCATACGAGATTCACTTCTGGACACAATCGCTTGC
B2	ACACTCTTTCCCTACACGACGCTCTTCCGATCT*NNNNNNNNNNN***MID**GGAGGTCTTGCAGACAGAGGA

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