DIANA
assay for inhibitor screening

Overview and collaboration proposal

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DNA-linked Inhibitor ANtibody Assay

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What is DIANA

- A **multi-well plate based assay** (with similar protocol to ELISA)
- Utilizing **detection probe consisting of small-molecule** active site ligand linked to reporter DNA oligo
- **Quantification by qPCR** with high sensitivity and broad dynamic range
- Can be implemented using standard equipment and be fully automatized - suitable for both academic labs and diagnostics / screening facilities

Two major application markets

1. **DIANA for diagnostics**
   Ultra-sensitive detection of active enzymes in range of clinical samples
   (zeptomoles of target protein can be detected, >7-log dynamic range)

2. **DIANA for screening**
   Screening for target enzyme inhibitors in drug discovery
   (sensitive hit discovery, quantitative, cost-efficient)

DNA-linked Inhibitor Antibody Assay (DIANA) for sensitive and selective enzyme detection and inhibitor screening. Navrátil *et al*. *Nucleic Acids Res* (**2017**); DOI: 10.1093/nar/gkw853

DIANA for screening: overview

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Application	• **Screening of compound libraries for new inhibitors / ligands** of target proteins • Successfully tested and validated on multiple target proteins and protein families - assays for new targets straightforward to develop[1]
Key advantages	• Extremely **high signal to noise ratio** (Z' > 0.9; CV < 5%) • **Quantitative**: compound K_i directly measured from a single well, hits ranked by inhibition potency, easy to screen for specificity in protein family • **Sensitive** : sensitive hit discovery, ultra-low false-positive and false-negative rate • **Robust**: works with unpurified protein, no interference or non-specific binding • **Cost-efficient:** very low compound consumption, allows for compound pooling leading to reduced screening costs and time
Potential customers	• **Pharma and CROs screening facilities:** automated HTS drug discovery • **Research institutions:** small-scale screens of in-house libraries against targets of interest (libraries of ~10k compounds can be screened in few 96-well plates)

1. Assuming at least one small-molecule inhibitor (even non-specific) exists and have suitable chemistry for modification.

DIANA for screening: 5-step protocol easy to implement in most screening facilities

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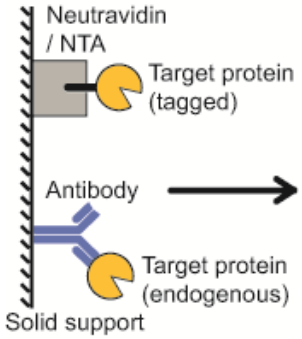
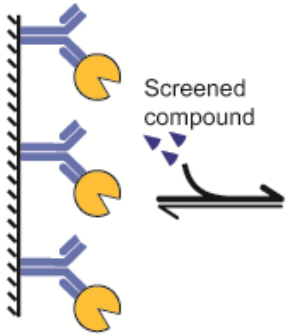
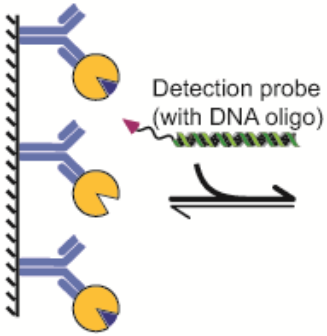
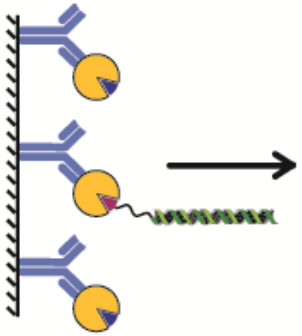
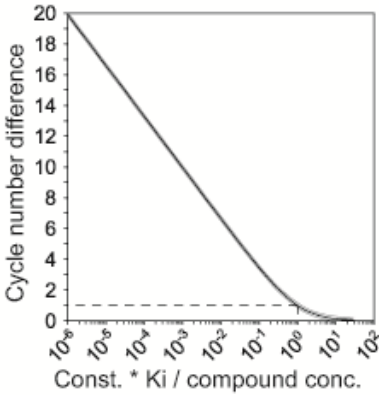
	1 Immobilization of target[1]	2 Incubation with compound[1]	3 Incubation with detection probe[1]	4 Washing[1]	5 qPCR detection
Protocol					
	• Target protein is captured in assay well via antibody or using affinity tag	• Screened small-molecule compound is added	• Detection probe (made of known inhibitor / ligand with attached DNA oligo) is added	• Unbound probe is removed by washing	• K_i of the compound calculated directly from qPCR cycle number difference to control wells without the compound
Setup	• Polypropylene qPCR compatible 96-, 384-well plates • Automated liquid handler / acoustic dispenser (ECHO)	• Automated liquid handler / acoustic dispenser (ECHO) • Small-molecule library to be screened	• Automated liquid handler / acoustic dispenser (ECHO) • Manual handling possible for small-scale experiments	• Most common washers (BioTek, BlueWasher) • Manual washing possible for small-scale experiments	• All common qPCR systems (Roche, ThermoFisher Scientific, Bio-Rad) • Standard qPCR evaluation software

1. No temperature sensitive incubations. Flexibility in incubation times. Tolerates high DMSO contents.

Key advantages: quantitative screening with broad dynamic range

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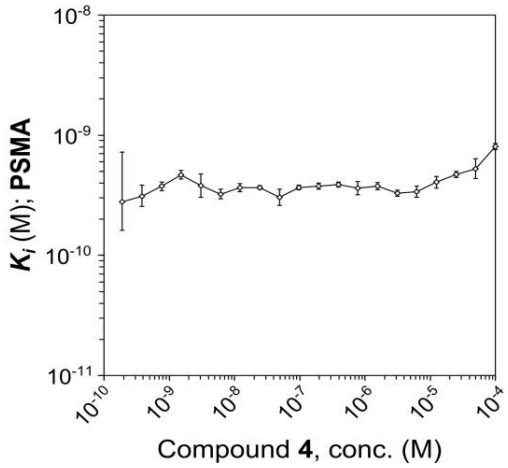
Quantification of affinity from a single read

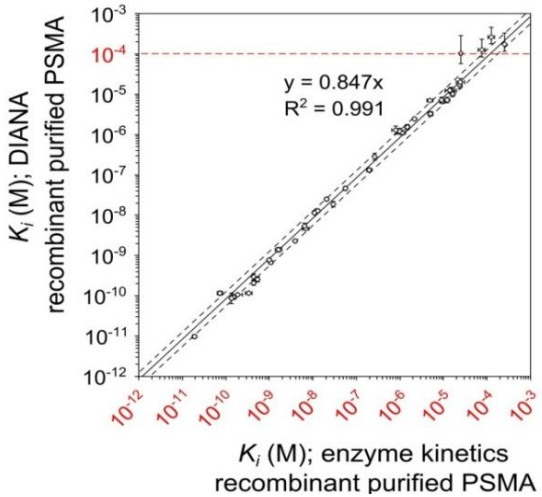
- **Accurate determination of K_i** from measurement at single concentration of tested compound
- Screening **hits ranked by the inhibition** potency
- Possible to test **specificity against whole protein families** in a single run



Purified PSMA standard was titrated with PSMA inhibitor. Measured inhibition constants were stable over more than 5 orders of magnitude of inhibitor concentration

Precise over broad concentration range

- Due to the qPCR detection, the same assay conditions can be used to precisely determine K_i of compounds **over ~7-log range** (e.g. 10pM - 100μM)
- DIANA measurements validated by orthogonal assays



K_i of 41 different compounds with 7-log difference in PSMA affinity were determined using the same 100 μM concentration. Measurements were validated by enzyme kinetics assays with serial dilution of inhibitors

Key advantages: ultra-sensitive and robust screening assay

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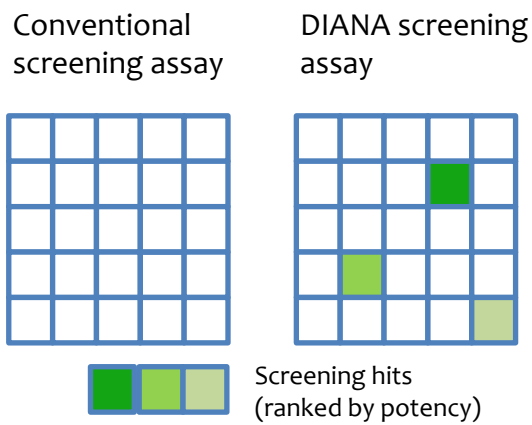
Higher sensitivity of detection

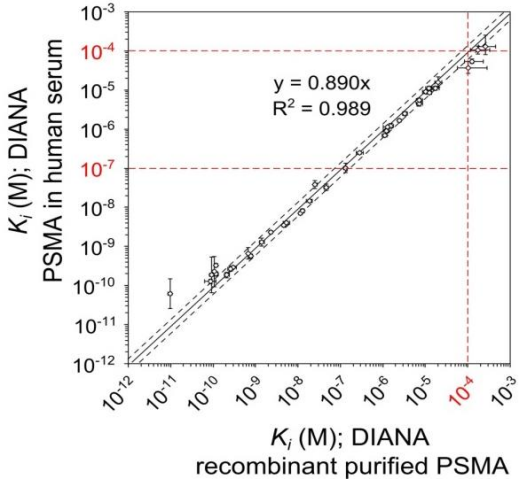
- Extremely **high signal to noise ratio** (Z'>0.9, CV<5%)
- **Sensitive in hit discovery** (IC_{50} = K_i)
- **Ultra-low false-positive** and **false-negative rates**
- **Easy counter-screen setup** to exclude false negatives



Due to high sensitivity and low background, hits are identified in DIANA screening even in libraries, which failed to produce any hits in conventional screening techniques.

Does not require recombinant protein

- Possible to screen inhibitors even with small amounts of targets endogenously present in body fluids – **no need for purified recombinant protein**
- Only **picograms of target protein** are sufficient to test the compound's activity[1]
- No fluorescent/colored compound interference



1 µl of human sera containing approx. 5 pg of endogenous PSMA was used for testing a compound's activity, with the same results as when using recombinant purified protein

1. Several orders less than what is usually required for ELISA or enzyme kinetics assays.

Key advantages: compatibility with pooled compound screen offering high cost-efficiency

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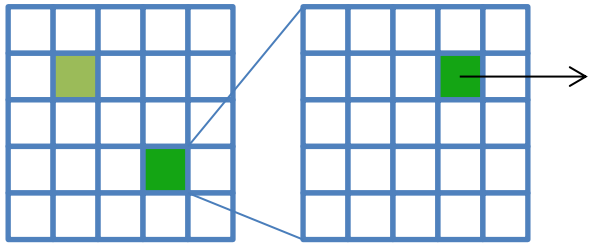
Compound pooling setup…

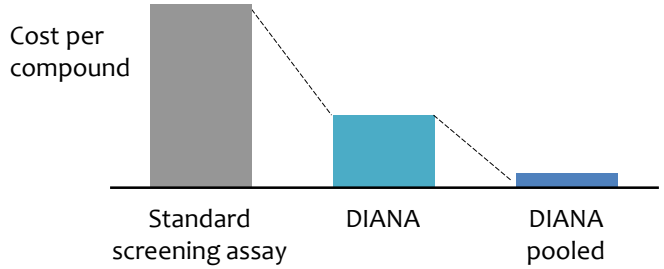
- **Compounds pooled prior to initial screening** - only wells with strong hits subsequently analyzed per individual compound
- Enabled by **quantitative** nature of the assay, ultra-high sensitivity (screening at low concentrations) and ultralow false positive and false negative rate
- ***Pilot study:*** strongest hits in a library of >2000 compounds identified using only two 96-well plates[1]



- ***In progress:*** screening of ~25,000 compounds in a single 384-well plate with 80 compounds per well

… leading to highly cost-efficient screening

- Significant **reduction of time required** to complete the screening project
- Unparalleled **low screening costs** (also considering low amount of protein and probe required)



- **Enhanced screening capabilities** even for small-scale academic and R&D labs:
  - Typical academic research library of **2000 compounds** can be screened for inhibition potency to target using a kit containing only **two 96-well plates**[1]
  - Small screening facility with a single 384-well plate qPCR machine is able to screen up to **~0.5 million compounds per day**[2]

1. First 96 wells-plate: initial screening run with 24 compounds per well. Second 96 wells-plate: subsequent analyses of 4 strongest pools for K_i of individual compounds
2. 16 plates per day (30 min qPCR) x 320 wells per plate x 80 compounds per well = 16 x 25600 = 409,600 compounds

Catalogue of targets: expanding range of assays for commercially relevant targets

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Status	Target	Relevant therapeutic area	Screening projects
Assay ready for screening	**Prostate specific membrane antigen (PSMA)**	• Oncology (Prostate cancer) • Neurology (CNS)	• Search for new scaffolds with desired pharmacokinetics
	Carbonic anhydrase family (CA-II, VII, IX, XII)	• Oncology	• Search for new specific inhibitors with desired pharmacokinetics
	Fibroblast activating protein (FAP)	• Oncology • Metabolic disorders	• Screen for new scaffolds
	FcγRI receptor, Glutamate carboxypeptidase III, Influenza neuraminidase, MTH1, …		
Priority pipeline	**Methyl transferases (e.g. EZH2)**	• Oncology • Epigenetics	• Screen for specific inhibitors (no off-targets)
	Hydroxysteroid dehydrogenase	• Woman health (Endometriosis) • Oncology (Breast cancer)	• Search for new inhibitor scaffolds
	Kinases	• Multiple therapeutic areas	• Pan-kinase probe allowing screening of inhibitors for any kinase target
	Insulin receptor family	• Hormonal disorders • Oncology	• Screen for specific ligands
On demand targets	Assay can be promptly developed on demand to majority of relevant protein targets[1]		

1. Assuming at least one small-molecule inhibitor (even non-specific) exists and have suitable chemistry for modification.

Our proposition: looking for partnerships to introduce DIANA to drug discovery market

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	Our proposition	Potential partners
1 **Assay development and licensing for screening projects**	• We **support implementation** of assay **at partner's site** and its adjustment to the screening project needs • We **synthesize the detection probe** and provide other reagents required • We can **develop DIANA based screening assay for any new target** of partner's interest	• Pharma screening center[1] • CRO[2] • Academic screening facility
2 **HTS screening services**	• We **screen partner's compound libraries** for hits to DIANA compatible targets and return quantification of their inhibition potency • We can run **mid- to high-throughput screening projects** at our facility (up to 10^6 compounds in a pooled compound setup)	• Pharma • Academic research groups[3] • Screening facility network
3 **Distribution of 'screening kits'**	• We **co-develop ready-made screening kits** and optimized instrumental setup – *see details in Appendix* • We **distribute via partner's product catalogue** to research laboratories and screening facilities for application in small-scale screening experiments	• Lab technology / chemical distributor • Instrument manufacturer

1. Non-exclusive licensing deal for screening the specific target existing with a major global pharma 2. Ongoing collaboration with European CRO 3. New inhibitor scaffolds already identified in in-house library screening project

Contacts for enquiries & other resources

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Contacts

Václav Navrátil – Head of R&D

- vaclav.navratil@uochb.cas.cz
- Methodology questions, assay development requirements

Jaromír Zahrádka & Martin Dienstbier – TTO

- zahradka@iocb-tto.cz, dienstbier@iocb-tto.cz
- Business questions, collaboration deals

Other contacts

- Martin Fusek, Jan Konvalinka

Further resources

Research paper

- Navratil et al. (2017): DIANA for sensitive and selective enzyme detection and inhibitor screening. *Nucleic Acids research*
- Screening methodology & Clinical diagnostics papers. *In preparation*

Introductory video

- www.youtube.com/watch?v=hrh82euICfU

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Appendix: DIANA-based kits for small-scale screening can be developed for range of targets

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Application	• **Screening of small- to mid-size compound libraries** for new inhibitors / ligands of target protein (research laboratories, academic and pharma screening facilities)
Kit content	• Target protein **coated 96- or 384-well plate** (*or* anti-GST or NTA coated + tagged target protein) • Target specific **detection probe** • Reconstitution buffers (for protein and probe) • 10x assay buffers (protein and probe dilution, wash)
Protocol and equipment required	• **Simple 3-step protocol:** incubation with compound and with probe in one step, washing, qPCR detection • Requires **standard lab equipment** and **multiwell-plate qPCR system** (automated liquid handler and plate washer are optional)